ECOPHYSIOLOGICAL STUDIES ON DEVELOPING EGGS AND OVIGEROUS FEMALES OF INTERTIDAL CRABS

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CONTENTS

ABSTRACT

Morphological, ecological and physiological adaptations of ovigerous crabs and their developing eggs to conditions on shore were compared in two species of New Zealand intertidal crabs.

Newly-laid eggs of both *Heterozius rotundifrons* and *Cyclograpsus lavauxi* are ellipsoidal, with initial mean volume of 204 and 10 nL respectively, increasing to 360 and 19 nL respectively before hatching. The incubation periods of eggs of *H. rotundifrons* and *C. lavauxi* were 194±3 and 56±1 days respectively at constant 15°C. Morphological criteria and timing of the 5 major developmental stages are reported: (1) Newly laid egg stage (2) Blastula stage (3) Gastrula stage (4) Eyespot and pigmentation stage and (5) Heart-beating stage. Two major membranes surround developing eggs at both early and late stages of *H. rotundifrons*. The thickness of the outer and inner membranes are approximately 3.4-4.8 and 0.6-0.9 μm respectively and are separated by a perivitelline space.

The timing of larval release was examined during continuous immersion (and continuing light/dark cycle) in ovigerous *H. rotundifrons* and *C. lavauxi* entrained to experimental tidal and light/dark cycles. For *H. rotundifrons*, larval release occurred over 4-6 consecutive days only during the dark phase at times related to both the light/dark and tidal cycles. For *C. lavauxi*, larval release was a single event occurring during high tide period at both light and dark phases. Detached eggs of both crab species were able to hatch into larvae independently of the female although the percentage hatching was low.

In *H. rotundifrons*, at 15°C, resting mass-specific $\dot{M}o_2$ of non-ovigerous and ovigerous crabs was of similar magnitude in air and water but for *C. lavauxi*, $\dot{M}o_2$ was about two times higher in air. In both species, in air and in water, the mass-specific $\dot{M}o_2$ of ovigerous crabs (adults and eggs combined) was higher than in non-ovigerous crabs, the difference being greatest in crabs with late stage eggs.

$\dot{M}o_2$ of developing eggs of *H. rotundifrons* and *C. lavauxi* was measured in seawater at 15°C. In both species, $\dot{M}o_2$ of eggs increased 10-12 times, the major increase being toward the end of their development. The cost of development, estimated from total O_2 consumption of single eggs of *H. rotundifrons* and *C. lavauxi*, was 1.517 and 0.077 μmol O_2 respectively. The 20-fold ratio is approximately equal to the ratio of their masses (about 19 times).

The elevated metabolic rates of ovigerous crabs can largely be accounted for by the increased metabolism of the eggs. However, a significant unexplained component of the elevation in *H. rotundifrons* suggests a small metabolic cost of bearing eggs.

In response to declining oxygen tension, eggs of *H. rotundifrons* and *C. lavauxi* exhibited characteristics of oxygen conformers and regulators respectively. The P_{crit} value for eggs at stage 5D of *C. lavauxi* was approximately 45 mmHg. The lactate concentration in eggs of *H. rotundifrons* increased from about 0.602 $mmol.L^{-1}$ in normoxic eggs to about 10.20 $mmol.L^{-1}$ after 3 hours hypoxic exposure. The rate of lactate accumulation during hypoxia suggests that the eggs are not sustaining metabolism by anaerobiosis, but are allowing metabolic depression.

In *H. rotundifrons*, no specific respiratory behaviour was associated with egg-bearing. Upon exposure to hypoxia, non-ovigerous and ovigerous crabs showed similar "emersion behaviour" (initial agitation, followed by leg extension and abdominal elevation and later quiescence). There was no difference in heart rates and scaphognathite activities between non-ovigerous and ovigerous *H. rotundifrons.* Both non-ovigerous and ovigerous crabs of this species showed decreased heart and ventilation rates in response to hypoxia which is similar to the behaviour found in other shore crabs.

Both early and late stage eggs of *H. rotundifrons* and *C. lavauxi* survived 24 hours exposure to dilute seawater and fresh water. Late stage eggs were more tolerant of reduced salinity than early stage eggs. Similar relationships existed when these eggs were acclimated for 96 hours to 50% seawater. In both species, eggs were hyperosmotic to the external medium in all salinities. The osmolalities of eggs were relatively insensitive to the external salinity and, even in fresh water, maintained osmolality equivalent to 70% seawater. Eggs exhibited remarkably little volume change over the same range of ambient salinities.

Measurements of four cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) in eggs at different stages after 24 hours exposure to a range of salinities, at 15°C, demonstrated that internal ions are retained and regulated differently at different egg stages. The overall concentrations of all four cations increased during development and decreased as salinities decreased. Sodium and potassium are the main cations in the eggs. Sodium was consistently hypo-ionic and potassium hyper-ionic compared with seawater. Sodium and magnesium concentrations changed at a proportionally greater rate with dilution than did potassium and calcium. These observations are consistent with location for most of the sodium and magnesium in a small compartment between the permeable outer egg membrane and the

embryo itself and with an embryonic (in the cells, blastocoel or yolk) location for most of the potassium and calcium.

Water and sodium influx studies suggested that egg membranes of *H. rotundifrons* are very permeable to water and salt throughout their development. Eggs at the early stage are more permeable to water but less permeable to sodium than eggs at the late stage. There are two components of water and sodium exchange in all egg stages of *H. rotundifrons*: a small rapidly exchanging or "fast pool" (outer pool) and a much larger, slowly exchanging or "slow pool" (inner pool). The outer pool is thought to represent the perivitelline space of eggs (4-8% of volume throughout development). The volume of exchangeable water in the inner pool increased with egg development.

Eggs at stage 2 and 5D acclimated to 50% seawater showed a permeability to water and sodium less than eggs acclimated to 100% seawater. Such a mechanism would be advantageous in an intertidal environment by limiting the work required to balance the osmotic influx of water and the loss of sodium.

The results of this study provided information on the ecology and physiology of ovigerous crabs and developing eggs and suggested that the eggs of both *H. rotundifrons* and *C. lavauxi* could survive periods of hypoxia that might be encountered when the female is partially buried at low tide or in shallow pools. Additionally, the eggs could cope physiologically with periods of dilution of the surface water by precipitation or freshwater seepage. Measurements of sodium and water fluxes indicated that, contrary to earlier suggestions, the eggs are highly permeable and are in dynamic exchange with the seawater at all stages.

CHAPTER ONE

GENERAL INTRODUCTION

Reproduction in decapod brachyurans is a complicated process which is influenced by internal and external factors. This process varies intra- and interspecifically, since it integrated with a variety of adaptations to the different habitats in which the animals live. These adaptations occur at the molecular, cellular, physiological, neurohormal and behavioral levels. Hence, this important and essential special event in the life of an animal influences population size, the distribution of species in time and space and finally determines the survival of each species in an ecosystem.

The process of reproduction in marine crabs begins with production of the gametes. However, the significant events start after fertilization. After fertilization, eggs are spawned and incubated underneath the abdomen of the female for a period of time before hatching. During this period embryos may encounter changes in environmental conditions which are outside their control. This is particularly the case in the tidal zone where there may be great variations in temperature, salinity and in the partial pressure of the dissolved gases. Thus, these environmental factors may play ecologically important roles in determining the survival of embryos within the eggs.

Development from a newly oviposited egg through a young pelagic larva is a continuous process. Egg development is sustained on the nutrient reserves in the form of proteinaceous yolk and lipid vesicles scattered throughout the cytoplasm. The protein yolk contains carotenoid pigments giving a characteristic coloration of eggs (Herring and Morris, 1975). The rate of egg development (incubation period) to a larva is ecologically important to the timing of larval release into the pelagic environment, and it may be influenced by both endogenous factors and interacting environmental factors. The endogenous factors regulating the rate of development of eggs may include genetic factors, composition of nutrient reserves, rate of substrate utilization for maintenance and tissue production, and size of eggs. The viability and rate of development of eggs are affected by a variety of environmental factors such as temperature, salinity, photoperiod and dissolved gases. The response of developing eggs to these environmental factors may change during the course of development. However, normal development to a larva takes place within a defined range of environmental conditions. When environmental conditions exceed the favourable range, the egg development is adversely affected, resulting in abnormal larva, cessation of development, or death of the embryo.

Many decapods are known to be able to regulate their body fluid concentration and gas tensions when faced with problems such as hypoxic conditions and hypo-salinity (Taylor, 1988). In adult crabs, the ability for physiological regulation in response to these environmental factors relies on the excretory system, gills and circulation. However little attention has been paid to the physiology of egg-bearing females. Do they still have the same regulatory ability? How can they protect their eggs from these unsuitable conditions? In eggs, since they are attached to the abdomen of their mothers, they also must experience variable environmental conditions. How do embryos manage themselves before their regulatory systems are developed? A single study (Wheatly, 1981) found that when oxygen tension of water is reduced to a critical level, the emersion response by parents is employed to raise the oxygen tension of the water surrounding the eggs. This behaviour is different from the typical emersion response found in males and unberried females in which the stream of bubbles emanated from the Milne-Edwards openings, which are the large openings at the base of the chelae. Instead the berried females were observed to reverse the direction of ventilation and direct a stream of bubbles out of the openings at the base of the posteriormost pair of walking legs and thus over the developing egg mass.

When intertidal organisms encounter salinity change, they must actively regulate their content of salt and water in order to avoid fatal dilution of their tissues. The effects of environmental salinity on the internal osmolality and specific ion regulation of adult decapod crustaceans have been investigated in numerous studies (Baldwin & Kirschner, 1976; Bursey & Bonner, 1977; Gérard & Gilles, 1972; Gérard, 1975; Gilles, 1970, 1977, 1978; Gilles & Gérard, 1974; Greenaway and Macmillan, 1978; Greenaway, 1988; Kévers, *et al.* 1979a,b; Lang & Gainer, 1969; Shaw, 1961; Smaldon, 1973; Spaargaren, 1974; Thompson & Pritchard, 1969b;). Developing eggs, however, have received little attention. Since at the early stage, eggs do not possess the differentiated organs and tissues employed by adults in osmotic-ionic regulation, how can these developing eggs protect themselves when there is a change in the concentration of the medium? In *Homarus gammarus*, Charmantier and Aiken (1987) found that intact eggs (embryos surrounded by both inner and outer membranes) were hyperosmotic to sea water and to dilute media. In contrast, hatchlings and prelarvae (embryos surrounded by the inner membrane only) were hyperosmoconformers (i.e. the osmotic pressure of the hemolymph is slightly higher than osmotic pressure of the medium within the ranges between 500-1100 mOsmol.kg^{-1}).

A short but critical period of reproduction, occurring at the end of the long incubation time, is the period of egg hatching or larval release. Timing of the hatching of eggs and larval release may involve endogenous factors within the egg and female parent, and interaction between endogenous and exogenous factors. A coordination of the processes within the egg and the parent, and with the environment may involve physiological and behavioural changes for synchronization of larval release. The control of hatching time in many decapod crustaceans has been studied and reported to occur rhythmically with respect to exogenous cycles such as phase of moon, tide and time of day (De Coursey, 1983; Forward, 1987). Rhythms in larval release also appear to be under endogenous control. It has been found that difference in the habitats of the adult crabs influences the control of larval release. Dominant control by the embryo occurs in sublittoral species, both the embryo and female control hatching in littoral species and control is primarily by the female in supralittoral species (Forward, 1987). Most crabs have planktonic larvae whose survival depends on many environmental factors such as water current, predation and food availability. Thus, the right time of larval release in these animals is another important factor determining the survival of their population.

The New Zealand "Big-handed Crab" *Heterozius rotundifrons* Milne Edwards, 1867 (Belliidae) and the "Smooth Shore Crab" *Cyclograpsus lavauxi* Milne Edwards, 1853 (Grapsidae) (Figure 1.1) are intertidal crabs found along the coastline of New Zealand. Although they are both intertidal crabs, they occupy different habitats. *H. rotundifrons* are usually found half-buried in sand beneath rocks in the lower half of intertidal area, whereas *C. lavauxi* are usually found under boulders in the upper half of intertidal area (Pellegrino, 1984). These two species of crabs are quite different in behaviour. The low-shore species is rather timid and unaggressive and, when disturbed, usually remains motionless, whereas the other species is much more active and often exposed to the air for long periods (McLay, 1988).

Although there have been many studies on the ecology and physiology of these two species of crabs (Innes *et al.*, 1986; Leader & Bedford, 1978; Pellegrino, 1984; Shanks, 1982; Waldron *et al.*, 1986), there is no information on the physiology of ovigerous females and their embryos. Ovigerous crabs of *H. rotundifrons* and *C. lavauxi* are of similar size (about 20 mm in cw and 2 g in mass) and carry similar masses of eggs (typically 0.2-0.3 g) on the abdominal pleopods. The number of eggs, however, are different. In *H. rotundifrons*, the brood consists of only a few hundred large yolky eggs (egg volume increasing from about 240 to 360 nL at hatching) which take about 6 months to develop and hatch in the laboratory at 15°C. In contrast, *C. lavauxi* carry several thousands of eggs (volume 9 to 19 nL) which hatch in only 2 months. Thus, the

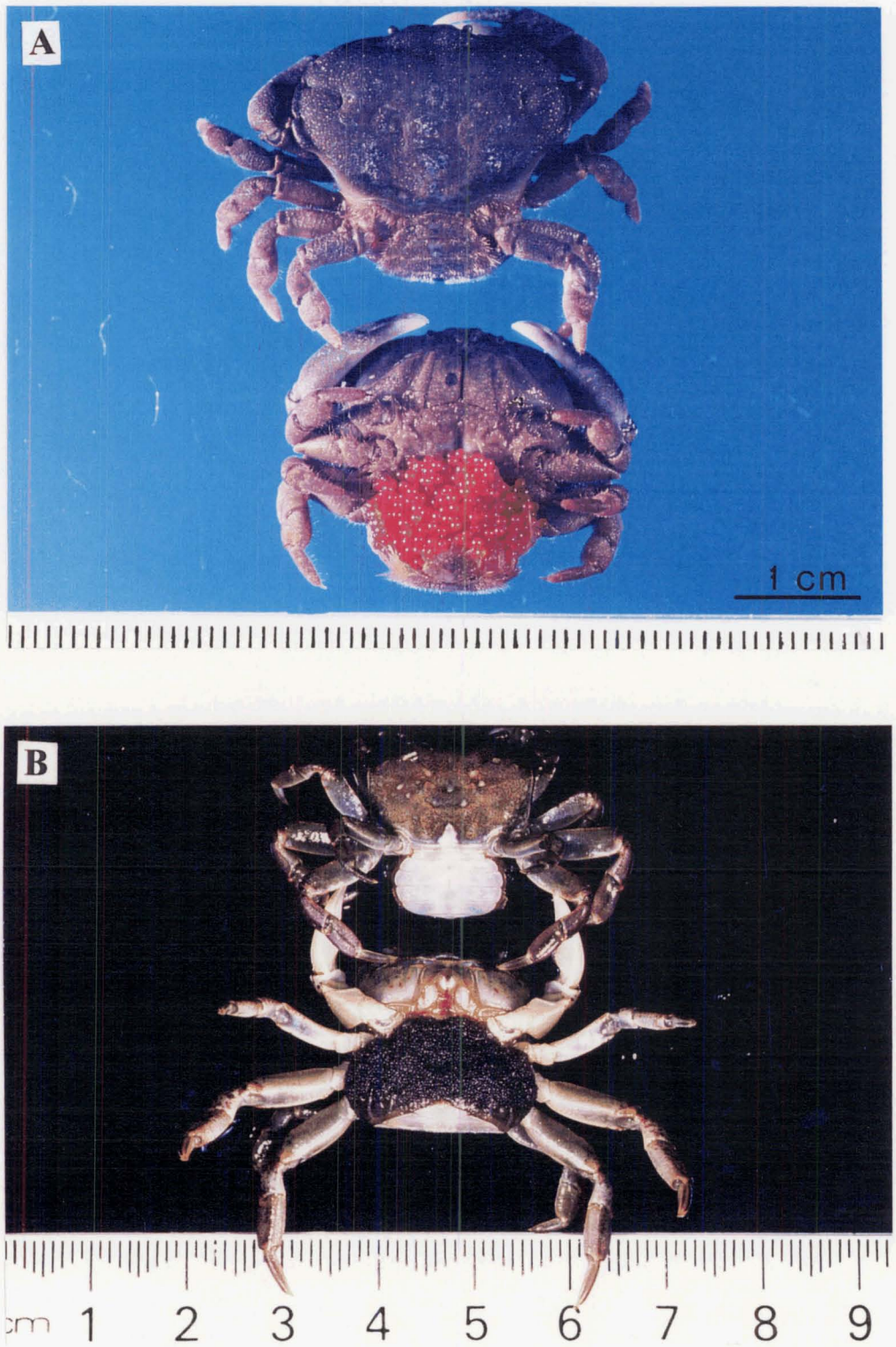


Figure 1.1. Non-ovigerous and ovigerous female crabs. A. *Heterozius rotundifrons* B. *Cyclograpsus lavauxi.*

developing eggs of both crabs, and especially of *H. rotundifrons*, have long incubation periods, and during this period, they must encounter quite severe changes in environmental factors. This leads to many interesting questions, e.g. Do the ovigerous crabs still have the same abilities in regulating their use of energy? Do different stages of eggs consume the same amount of oxygen during their development? Is there any relationship between oxygen consumption of females and their embryos? Do developing eggs of each species have the same or different abilities in osmotic and ionic regulation?

SCOPE OF THE STUDY

The aim of this thesis was to study the ecological and physiological adaptations of ovigerous crabs and their developing eggs to conditions on the shore comparing *Heterozius rotundifrons* and *Cyclograpsus lavauxi* throughout the period of egg development (from spawning to hatching). Chapter 2 documents the embryonic developmental stages and incubation periods of the eggs. Morphological changes that define the embryonic stages and time required to complete the development were studied and compared in the laboratory at constant temperature (15°C). These studies define the stages used in other physiological experiments. The internal structure of the egg and its membranes of *H. rotundifrons* was also examined in this chapter using optical and transmission electron microscopy. Experiments on the timing of egg hatching are reported in chapter 3. Ovigerous crabs of both *H. rotundifrons* and *C. lavauxi* with prehatching eggs were entrained to artificial tidal and light/dark cycles and the timing of larval release was then examined during several days of continuous immersion (and continuing light/dark cycle). It will be shown that for *C. lavauxi*, larval release is a single event, is independent of the light cycle and phase-locked to the previously entrained tidal cycle. *H. rotundifrons*, by contrast, releases larvae in several bursts which appear to be related to both the time of day and the tide. The demonstration of release rhythms in the absence of a direct tidal cue raised the question of their control. Thus, initial experiments comparing the hatching of eggs detached and isolated from the female with those still attached are also included in this chapter.

Chapters 4 and 5 concern the physiological study of respiration of ovigerous crabs and developing eggs. Chapter 4 provides information on the overall oxygen requirements in air and water of ovigerous females of both *H. rotundifrons* and *C. lavauxi*. Oxygen consumption by the separated eggs at different developmental stages was also measured in order to assess the component of total respiration attributable to the eggs. The measurements of oxygen consumption at timed intervals during development from

spawning to hatching were combined to estimate the total cost of development in terms of oxygen uptake for these two species of differing egg size and development time. The effects of changes in Po_2 on heart beat and scaphognathite activity of non-ovigerous and ovigerous *H. rotundifrons* are examined in chapter 5. A comparison is made of the effects of a decrease in Po_2 on the oxygen consumption of eggs at different stages of *H. rotundifrons* and *C. lavauxi*. Finally, in this chapter, the accumulation of lactic acid during hypoxia of *H. rotundifrons* eggs is reported.

A study of osmoregulation of the eggs is reported in chapter 6 and 7. The range of salinity tolerance of eggs from both *H. rotundifrons* and *C. lavauxi* at different developmental stages is documented in chapter 6. In this chapter, whether the eggs of both species osmoregulated or osmoconformed was examined by studying the time course of changes in internal osmolality, of total volume, of solvent volume and of the concentrations of the cations Na^+, K^+, Ca^{2+} and Mg^{2+}. It will be shown that at all stages the eggs of both species maintain a hyperosmotic condition in dilute seawater for long periods. Whether this was simply a result low permeability and slow equilibration, or a true dynamic steady state, was investigated further in chapter 7 by measurement of fluxes of labelled water and sodium in *H. rotundifrons* eggs. The kinetics of water and sodium exchanges in this chapter provide further information on their compartmentalization within the egg and the location of likely permeability barriers.

CHAPTER TWO

EMBRYONIC DEVELOPMENT AND MORPHOLOGY OF CRAB EGGS

INTRODUCTION

Female brachyuran crabs, after spawning, incubate their fertilized eggs by attaching them to the abdominal appendages. Embryos within the eggs then gradually develop with time using the energy of the yolk accumulated inside the eggs, and finally emerge as larvae which pass through a series of moults before assuming the adult form.

Crab eggs show great variations in colour, size and shape. In general the shape is spheroidal, with the yolk concentrated as "yolk pyramids" (Figure 2.1) in the interior of the egg, and the cytoplasm is distributed as a thin coat on the external surface (Richards, 1931 and Balinsky, 1981). The amount of yolk in the egg is directly related to the egg's size which appears to vary among crab species. Amongst Crustacea, the larger the eggs, the longer is the duration of embryonic development (Steele & Steele, 1975). The duration of egg development in crabs from spawning to hatching is influenced by many factors. Wear (1974) found that during embryonic development, egg volume increases, the rate of increase being slower in the eggs of species with a long development period than in those that develop rapidly. The incubation period in decapod crustaceans has been reported to be influenced by temperature (Branford, 1978). In brachyuran, *Macropipus depurator*, a threefold decrease in egg development time can occur naturally during the early spring to mid-summer breeding season (Wear, 1974).

There have been few studies of embryonic development in decapod crustaceans. Herrick (1896) described development of the eggs of the lobster, *Homarus americanus*, while Boolootian *et al.* (1959) and Lawinski & Weglarska (1959) described morphological changes in the eggs of several brachyuran and anomuran crabs. Within the genus, *Callinectes*, Uhler (1976) and Rogers-Talbert (1948) correlated gross changes in the colour of the egg mass of *C. sapidus* with the apparent age of the eggs. Perkins (1972) working with *Homarus americanus*, attempted to construct an index to monitor the rates and degree of embryo development. Recently, Helluy & Beltz (1991) studied the embryonic development of *H. americanus* using a quantitative staging system in which the sequence of appearance of morphological, anatomical, and behavioural characteristics were observed.

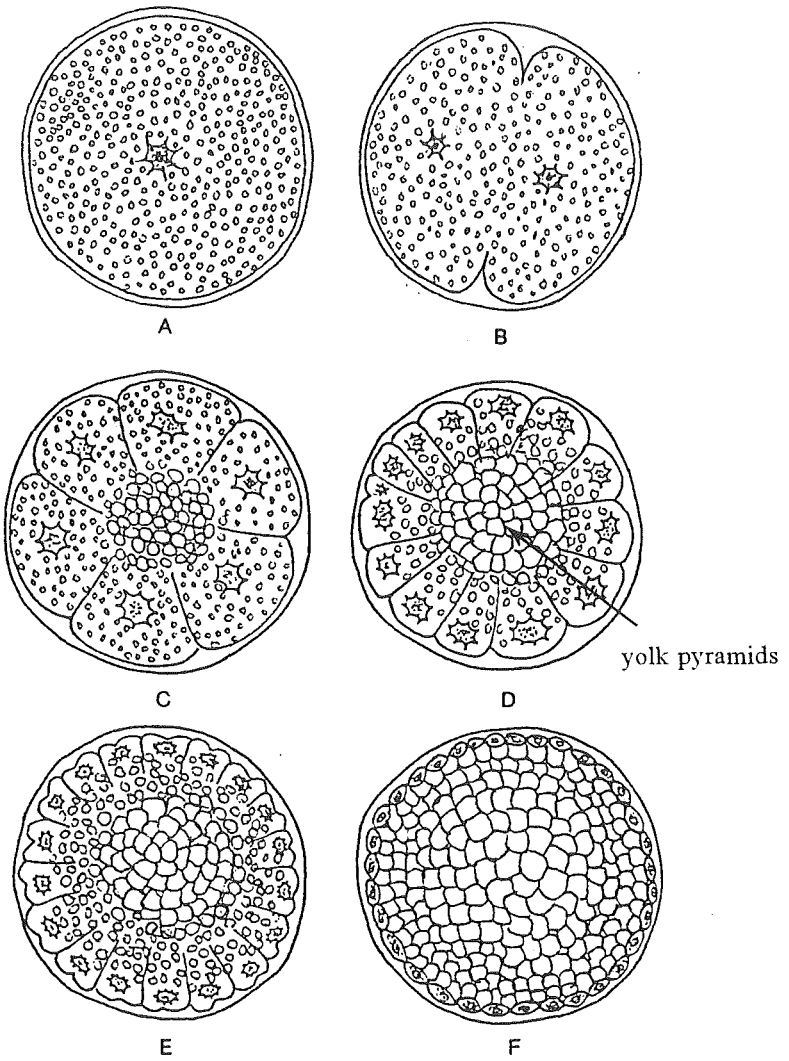


Figure 2.1 Development of a crab egg, showing cleavage cells, the yolk pyramids and the blastoderm. Newly laid egg (A), Cleavage (B-E) and Blastula stage (F) (Redrawn from Richards, 1931).

The "Big-handed Crab" *Heterozius rotundifrons* A. Milne Edwards, 1867 and the "Smooth Shore Crab" *Cyclograpsus lavauxi* H. Milne Edwards, 1853 belong to the family Belliidae and Grapsidae respectively. *H. rotundifrons* is endemic to New Zealand (McLay, 1988). Both species are found along the coastline of New Zealand. Many aspects of the ecology and physiology of these two species of crabs have been studied including their reproductive biology (Jones, 1978; Olsen, 1992). At Kaikoura, ovigerous females of *H. rotundifrons* are found throughout the year except in February (Jones, 1978). Jones (1978) reported that females carrying newly deposited eggs are found from March to October and those carrying final stage eggs are found from August to January. He proposed that for this species at Kaikoura there are two broods with a maximum incubation time of approximately 5 months in winter and a minimum incubation time of 3 months in summer. However, Wear (1968) suggested that the incubation period for *H. rotundifrons* at Wellington was at least 5 months. From the data given by Steele & Steele (1975), eggs of *H. rotundifrons* would be expected to have an incubation time of 105-160 days at 10°C. Steele & Steele (1975) suggested that this species should have an extended incubation period, since they have large eggs. From my field observation at Kaikoura, I found no evidence of two brooding cycles at this location. *H. rotundifrons* started spawning around late-March until early-May and hatching occurred around mid-December until late-January.

Compared with *H. rotundifrons*, ovigerous females of *C. lavauxi* have smaller eggs. Dell & Marshall (1967) reported the occurrence of ovigerous females of this species at Whakatane in December and at Wellington from September to January but they found that the peak of the breeding season extends from mid-October to late December. Thomson and Anderton (1921) reported that eggs hatch in December and January in Otago Harbour. Olsen (1992) studied the reproductive biology and population ecology of *C. lavauxi* in the Avon-Heathcote Estuary and found that the breeding season is extended from November to January/February and requires an average of 45 days for egg development under laboratory conditions at 16°C. At Kaikoura, females carrying eggs ready to hatch have been found in February (Wear, 1970). From my field observation at Kaikoura, I found that *C. lavauxi* started spawning around mid-November and the final stage eggs were found between mid-January to mid-February.

From the above observations, it can be seen that, on the shore, the developmental stages and incubation periods of both *H. rotundifrons* and *C. lavauxi* are variable and there are still no data available on the structure and volume change during egg development. In this study, morphological changes that define the embryonic stages and the time required to complete the development by crab embryos were studied and compared between *H.*

rotundifrons and *C. lavauxi* in the laboratory at constant temperature (15°C). The principle criteria used to identify the developmental stages were based on the changes in yolk content, relative size of the embryo, eye development, embryonic pigmentation and rhythmic heartbeats (Perkins, 1972, Helluy & Beltz,1991). Additionally, in this study, the internal structure of the egg and its membranes of *H. rotundifrons* was examined using transmission electron microscope (T.E.M.). The development of eggs of both crab species were carefully investigated from the time they were spawned until hatching. The data on embryonic structure, timing of main growth periods, yolk utilization, volume changes and membrane structures of these eggs were then used to characterize stages of eggs used in metabolic ($\dot{M}o_2$) and osmo-ionic studies described elsewhere in this thesis.

MATERIALS AND METHODS

Maintenance of ovigerous crabs and tidal tank system

Non-ovigerous females of *H. rotundifrons* and *C. lavauxi* were collected from natural habitats along the intertidal zone at Kaikoura (Kean point car park, Figure 2.2) during the period of spawning. The collected crabs were then transferred to Department of Zoology, University of Canterbury and reared in a recirculating tidal tank system in a controlled temperature room (15°C) under a 12h light and 12h dark cycles. The salinity of seawater was maintained between 33 and 35 ppt throughout the study period. A layer of beach gravel 2-3 cm thick was arranged on the bottom of the tank to expedite the attachment of the eggs to the pleopods and to prevent egg loss, which occurs quite frequently in plastic bottomed tanks (Edwards, 1979). As female crabs spawned, they were removed and reared in separate recirculating tidal tanks in the same controlled temperature room. Five ovigerous females were kept in each tank for embryonic developmental and morphological studies of their eggs. To be recognized, a numbered plastic tag was glued to the carapace of each ovigerous female using cyanoacrylate glue. Ovigerous females were fed once a week with chopped mussel and the seawater in the tank was changed every week.

The tidal systems provided alternate periods of immersion (high tide) and emersion (low tide) of equal duration (6h 12 min). Each consisted of two tanks, a filter, a small pump and two household timers. Two tanks consisted of polypropylene stacking fish boxes of similar base dimensions but of different heights. The smaller tank, 40.5 cm wide × 63.5 cm long × 21.5 cm high was used for holding crabs and rested on the larger tank (40 cm high) which was used as a reservoir. A plastic filter covered with a layer of gravel, 5 cm thick, was placed on the bottom of the reservoir tank. An air flow system was connected to the filter and this aerated and conditioned the seawater. High and low water were controlled by a small pump placed inside the reservoir tank. The operation of the pump was controlled by the two timers. Timer 1 (an electromechanical clock and relay) switched the pump on and off for, nominally, 6h periods. The timer 2 (an electronic clock and relay with battery backup) was set to control the tidal delay time which is 48 min per day in this tidal system (about 50 min per day on the shore naturally). This was achieved by using timer 2 to interrupt the power supply to the clock of timer 1 for 12 periods of 4 min per day. When the pump turned on, seawater in the reservoir tank was pumped into the header tank and flowed back down to the reservoir tank through two overflow pipes (the pump output exceeded the flow in the

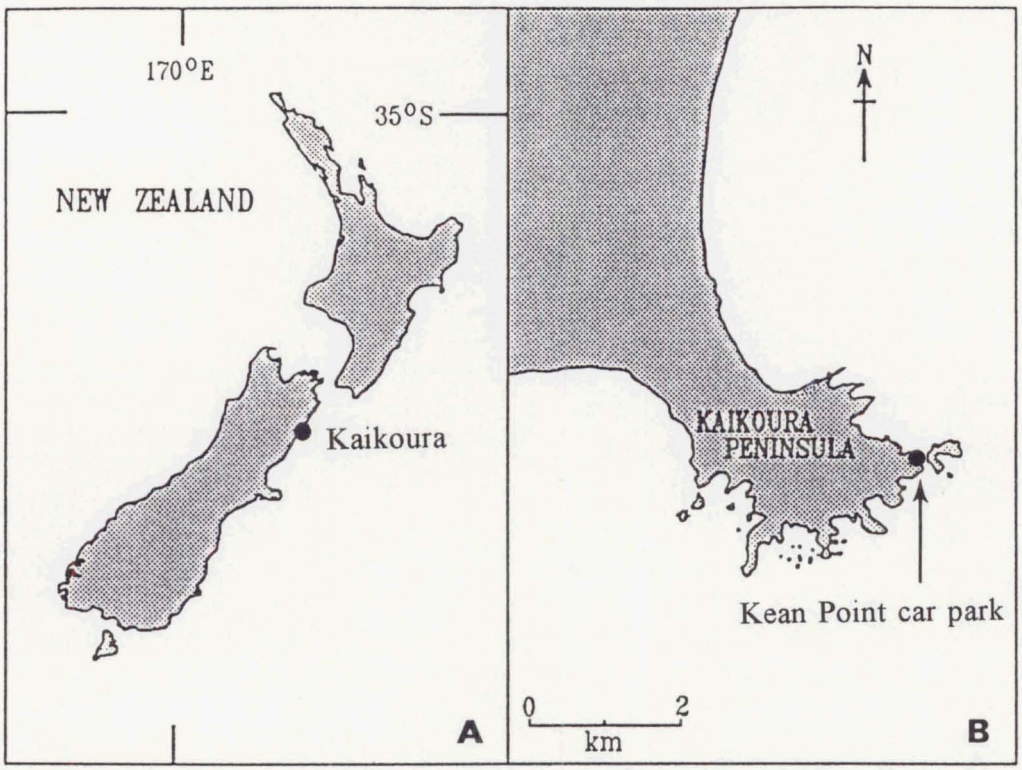


Figure 2.2 Location of the site of collection of crabs at Kaikoura (A) Map of New Zealand (B) Kaikoura Peninsula showing the location of Kean Point car park close to the area used for collecting crabs (C) View from Kean Point car park at Kaikoura.

lower pipe) (Figure 2.3A). When the pump was switched off, the system drained back into the lower reserviour through the lower overflow pipe and no circulation of seawater occurred (Figure 2.3B). However, at low water, some interstitial water was retained by the gravel.

Embryonic development and incubation period of crab eggs

To study embryonic development, five ovigerous females of each species were examined weekly (3-5 days for *C. lavauxi*) from spawning to hatching. The developmental stage, based on morphological criteria was recorded and the duration of embryonic development was determined. At each sample time, five eggs were removed from each ovigerous crab and observed through a stereo-microscope. The following features were noted: egg diameter, eye diameter, quantity of yolk, colour of egg mass, appearance of embryonic larval chromatophores and heart beat. Measurements were made using an ocular micrometer at a magnification of 80x. Photographs of each stage were taken using a camera mounted on a stereo-microscope to identify and compare the different stages.

Egg volume was calculated from the average diameters of the eggs according to the revolution ellipsoid formula, $V = 4/3\pi(L/2)(l/2)^2$ where "L" and "l" are the greatest and smallest diameters respectively (Valdes *et al.*, 1991). The "eye index" was determined as an average of its greatest width and greatest length. This measurement was used as a reference for development only and was not meant to represent the actual increase or growth of the eye (Perkins,1972).

The volume of yolk remaining in the eggs at various stages in development was obtained as the sum of the volumes of a number of imaginary, regularly shaped solid bodies which when fitted together would give good approximation to the irregular yolk mass (Wear, 1974). This was expressed as a fraction of the original egg volume. Thereafter it was possible, by comparison, to estimate visually the amount of yolk remaining at each stage.

Observation on characteristics of the egg membrane of H. rotundifrons

Characteristics of the egg membranes of *H. rotundifrons* were studied using the stereo-microscope and the transmission electron microscope. Developing eggs were collected

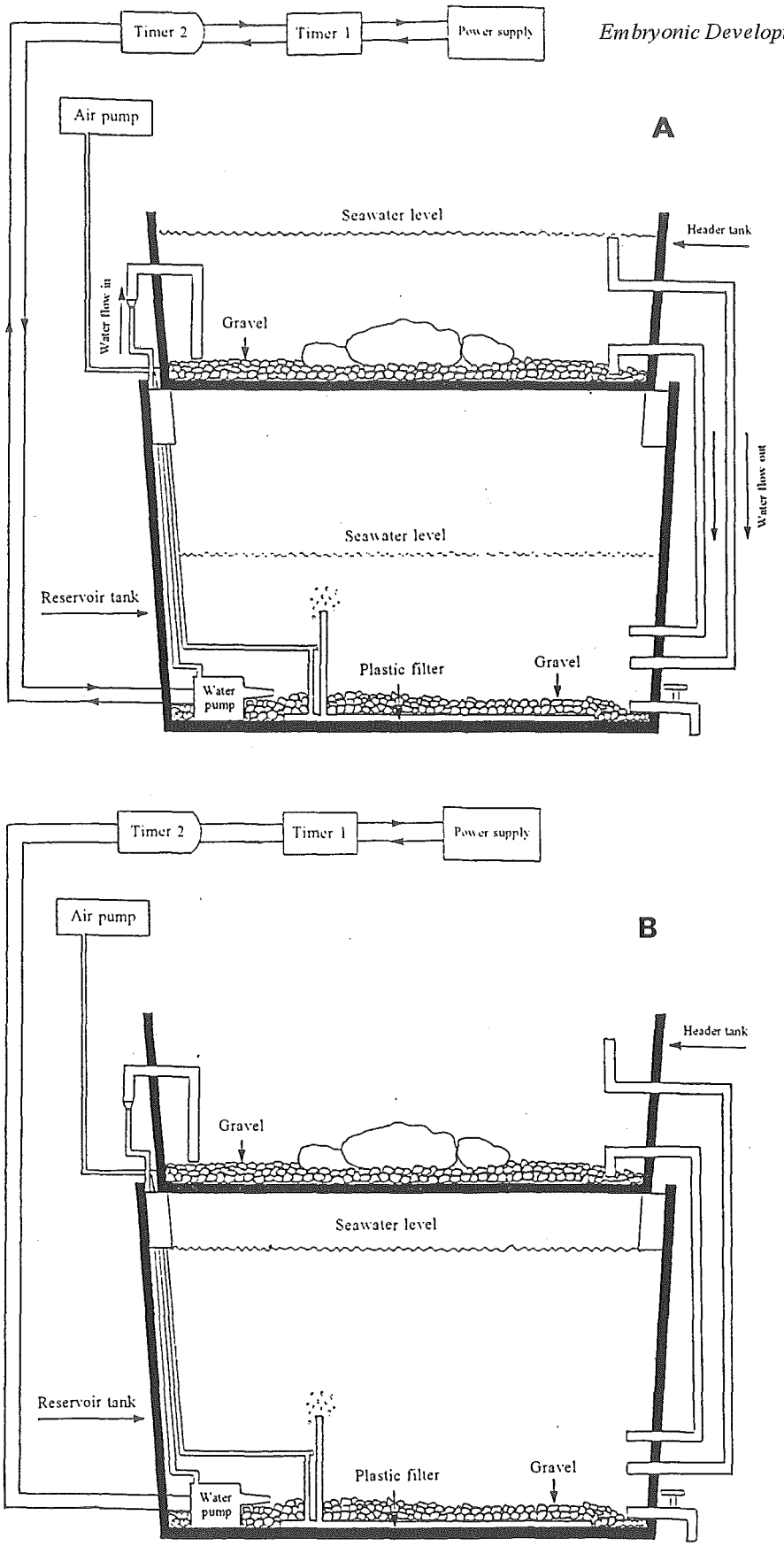


Figure 2.3 Schematic diagrams of tidal tank system (A) During high tide period (B) During low tide period

from the pleopodal setae of the ovigerous females, using forceps. They were fixed primarily with 1% formaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (made up in seawater; pH 7.4) for 8 hours and rinsed in the buffer for 30 min at 4°C. Specimens were then post-fixed in 2% buffered osmium tetroxide overnight and rinsed again in the same buffer for 20 min. After gradual dehydration in ethanol and acetone, the eggs were infiltrated in Spurr's embedding medium on a rotary infiltrator. The 100% Spurr's medium was replaced with fresh medium before the samples were polymerized in plastic moulds at 70°C for 24 hours. Thin sectioning was accomplished using an LKB 8800 ultramicrotome equipped with a glass knife. Gold sections were stained with 5% uranyl acetate, and followed by lead citrate stain and then examined with JEOL 1200EX transmission electron microscope, at an accelerating voltage of 80kV.

RESULTS

General morphological observations

Newly laid eggs of both *H. rotundifrons* and *C. lavauxi* were seen as masses of undifferentiated material, with the yolk occupying 100% of the egg space. They had slightly elliptic shapes and were easily deformable. Each egg was enclosed in a tough outer membrane which extended as a conical projection (funiculus) which attached it to one of the pleopods. The patterns of embryonic development in eggs of two species of crabs considered in this work were similar, with yolk cleavages occurring a few days after spawning, followed by invagination, gastrulation, tissue formation of organs and finally hatching as a zoeal stage. The order of appearance of some morphological features such as chromatophores, black eye pigment and the first indications of heartbeat did not vary from *H. rotundifrons* to *C. lavauxi*, although there were some differences in the percentage of the original yolk volume metabolized relative to the degree of development of the embryo. In *H. rotundifrons*, which has very large eggs, these characters appeared with about 30% of the original yolk volume metabolized compared with about 50% in the very small eggs of *C. lavauxi* (Table 2.1 and 2.2).

At 15°C, developing eggs of *H. rotundifrons* and *C. lavauxi* increased their volume significantly during embryonic development. The percentage volume increase of eggs during development related to their original egg volume were 76.96% and 83.65% in *H. rotundifrons* and *C. lavauxi* respectively (Table 2.3). Graphs plotted between development time and egg volume showed similar patterns of development between eggs of both species (Figure 2.4 and 2.5). According to the rate at which egg volume increased, development curves of both species can be divided into two phases, a slow phase which occurred during blastulation and a fast phase which occurred after gastrulation. Although the overall pattern of egg development of both species was similar, there were some differences between the two developmental curves. The main difference appears to be that, in *H. rotundifrons*, the rapid increase in egg volume occurs about two-thirds of way through the development, whereas in *C. lavauxi* it is about one-third. In *H. rotundifrons*, the egg volume was relatively constant during the blastula stage before the beginning of gastrulation. During gastrulation, embryos started forming tissue caps, eyespots and chromatophore pigments and egg volume increased only slowly, then rapidly after the appearance of the heartbeat. The regression line of the relationship between eye index and development time of *H. rotundifrons* is Y = 2.9803 (x) - 292.9569 (Figure 2.6). In *C. lavauxi*, during the blastula stage, the egg volume was

Table 2.1 Average (mean ± SE) of egg width, egg length, egg volume, quantity of yolk and development time at each stage of developing eggs of *Heterozius rotundifrons*, at 15° C

Stage	Egg width (mm)	Egg length (mm)	Egg volume (mm^3)	Quantity of yolk	Duration (d)	Total time elapsed to end of stage (d)
1. Undeveloped egg and early cleavage	0.712 ± 0.008	0.768 ± 0.008	0.204 ± 0.006	full yolk	6.8 ± 0.8	7
2. Morula and blastula	0.743 ± 0.016	0.794 ± 0.019	0.230 ± 0.009	full yolk	48.8 ± 3.2	56
3. Gastrula and germ layers	0.762 ± 0.015	0.809 ± 0.013	0.246 ± 0.010	yolk 9/10-5/6	48.2 ± 3.1	104
4. Eyespots and pigmentation	0.781 ± 0.016	0.827 ± 0.017	0.264 ± 0.012	yolk 5/6-4/5	25.4 ± 1.1	129
5A. Beginning of heart beat	0.803 ± 0.015	0.847 ± 0.017	0.287 ± 0.015	yolk 4/5-3/5	19.6 ± 1.1	149
5B. Yolk in four big lobes	0.842 ± 0.017	0.879 ± 0.019	0.327 ± 0.016	yolk 3/5-1/3	19.0 ± 0.7	168
5C. Yolk in two big and two small lobes	0.868 ± 0.014	0.901 ± 0.016	0.355 ± 0.013	yolk 1/3-1/4	19.2 ±1.9	187
5D. Yolk in two lobes (before hatching)	0.874 ± 0.012	0.903 ± 0.015	0.361± 0.010	yolk in two lobes	6.8 ± 0.84	194

Table 2.2 Average (mean ± SE) of egg width, egg length, egg volume, quantity of yolk and development time at each stage of developing eggs of *Cyclograpsus lavauxi*, at 15° C

Stage	Egg width (mm)	Egg length (mm)	Egg volume (mm^3)	Quantity of yolk	Duration (d)	Total time elapsed to end of stage (d)
1. Undeveloped egg and early cleavage	0.262 ± 0.006	0.288 ± 0.005	0.0104 ± 0.0006	full yolk	4.0 ± 0.7	4
2. Morula and blastula	0.263 ± 0.009	0.289 ± 0.006	0.0105 ± 0.0009	full yolk	7.4 ± 0.5	11
3. Gastrula and germ layers	0.274 ± 0.012	0.300 ± 0.012	0.0118 ± 0.0015	yolk 9/10-1/2	21.0 ± 2	32
4. Eyespots and pigmentation	0.304 ± 0.013	0.326 ± 0.016	0.0158 ± 0.0020	yolk 1/2-1/4	9.6 ± 1.1	42
5 (A-C). Heart-beating	0.316 ± 0.007	0.337 ± 0.007	0.0176 ± 0.0011	yolk in four lobes	12.4 ± 1.7	54
5D. Before hatching	0.325 ± 0.009	0.345 ± 0.008	0.0191 ± 0.0006	yolk in two lobes	2.0 ± 0	56

Table 2.3 Average (mean ± SE) of development time, original egg volume, egg volume at hatching and percentage volume increase of developing eggs of crabs, *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, at 15°C.

Species	No. of crabs	Development time (day)	Original egg volume (mm^3)	Egg volume at hatching (mm^3)	% volume increase
H. rotundifrons	5	194 ± 3	0.204 ± 0.006	0.361± 0.010	76.96
C. lavauxi	5	56 ± 1	0.0104 ± 0.0006	0.0191± 0.0006	83.65

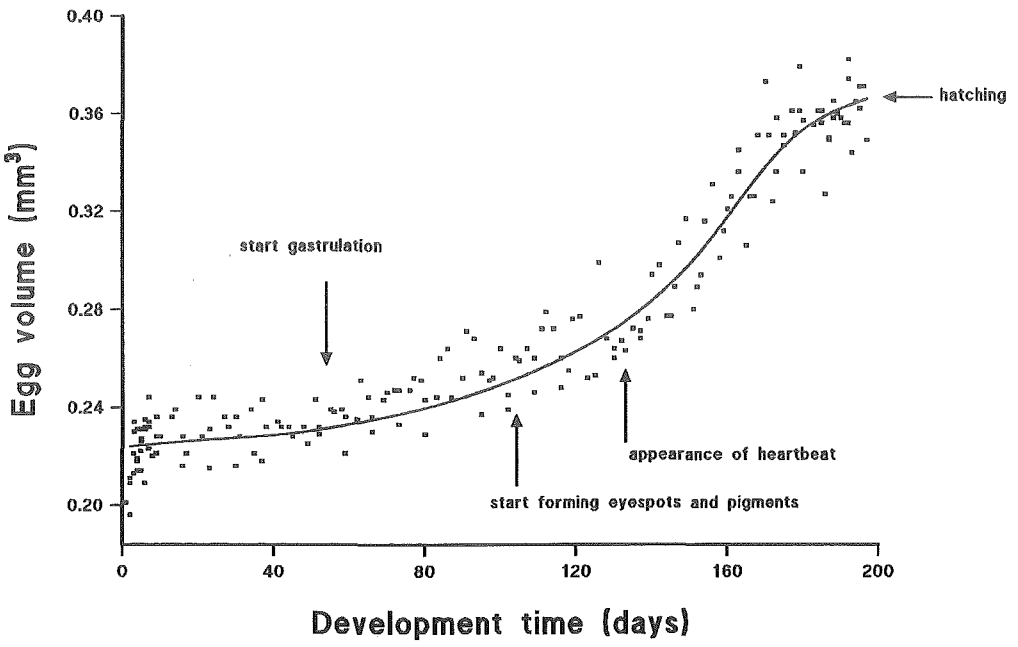


Figure 2.4 The relationship between development time and egg volume of *Heterozius rotundifrons*, at 15°C.

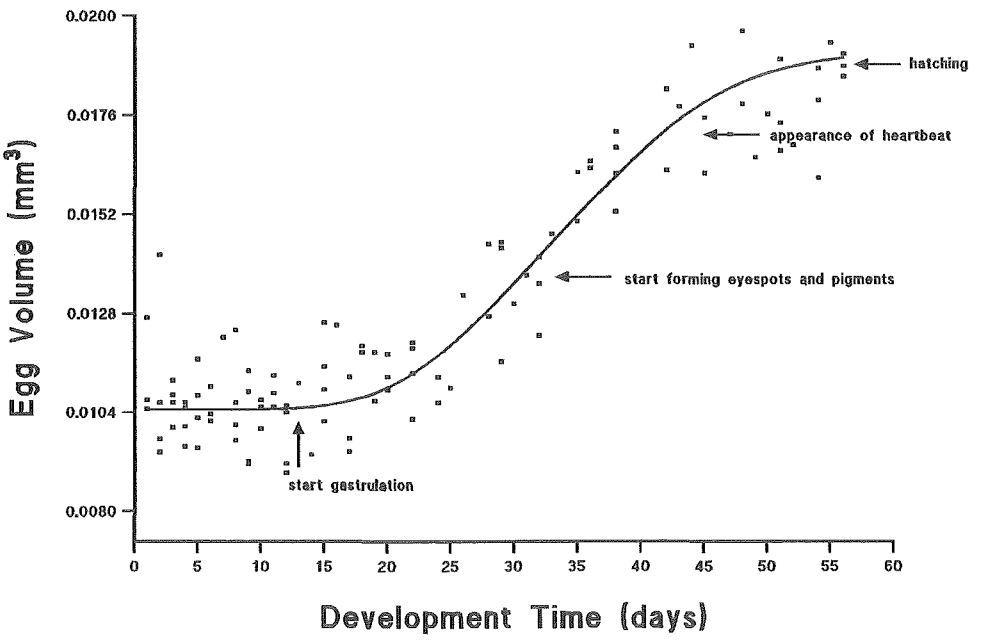


Figure 2.5 The relationship between development time and egg volume of *Cyclograpsus lavauxi*, at 15°C.

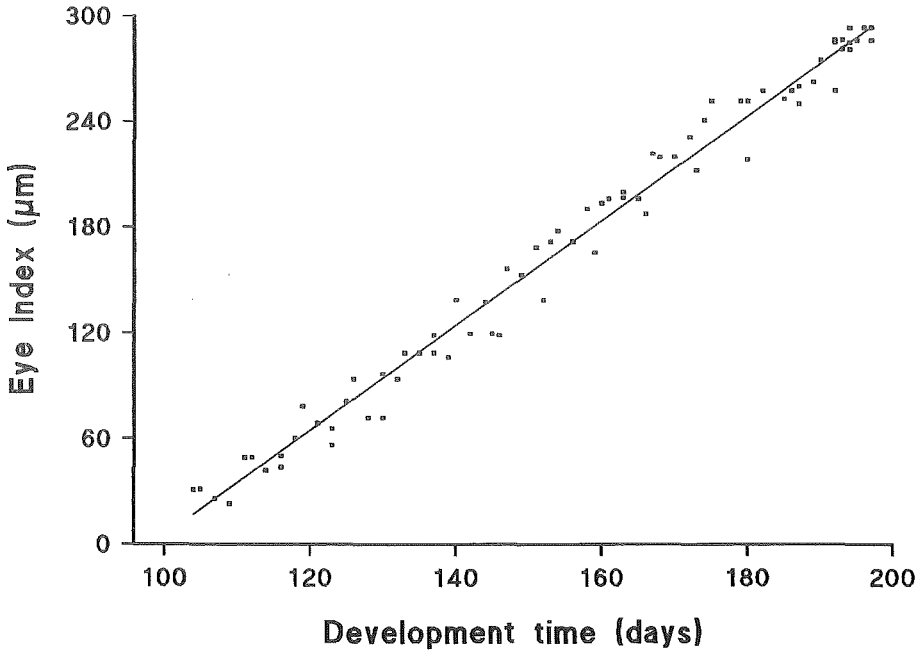


Figure 2.6 The relationship between development time and eye index of *Heterozius rotundifrons*. The regression line is Y = 2.9803 (x) - 292.9569, r = 0.99 (n = 76).

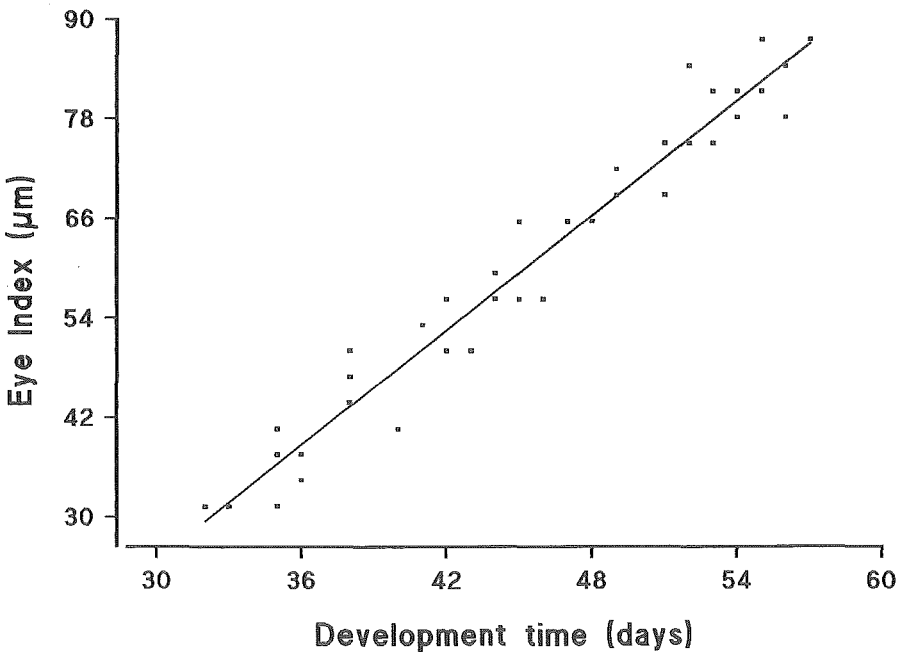


Figure 2.7 The relationship between development time and eye index of *Cyclograpsus lavauxi*. The regression line is Y = 2.3034 (x) - 44.2560, r = 0.98 (n = 45).

relatively constant, then it increased very rapidly after gastrulation and this time the embryo formed eyespots and chromatophore pigments including the first appearance of the heartbeat. The regression line of the relationship between eye index and development time of *C. lavauxi* is Y = 2.3034 (x) - 44.2560 (Figure 2.7).

Embryonic development of Heterozius rotundifrons

The eggs of *H. rotundifrons* were large and yolky. Newly-laid eggs were ellipsoidal and during development they became more spherical. Mean diameters of newly-laid eggs were 0.71 x 0.77 mm. (i.e. a volume of 0.204 mm^3 or 204 nL). With the progress of development, the eggs increased in size and before hatching, they averaged 0.87 x 0.90 mm. (i.e. a volume of 0.361 mm^3 or 361 nL).

At 15°C, the embryos inside the eggs of *H. rotundifrons* were incubated under the abdomen of their mother for a period of 194±3 days. A few days after spawning, the eggs began differentiation with yolk cleavage followed by invagination, gastrulation, tissue formation and finally hatching as a zoeal stage. The colour of the egg mass changed from orange-red at the beginning to dark-red, dark-brown and finally dark-black at the end of development just prior to hatching. The embryonic stages can be conveniently defined into 5 stages, based on the changes in morphological and physiological characteristics (Table 2.1 and Figure 2.8).

Stage 1. Undeveloped eggs and cleavage stages. (1-7 days) Starting after the egg was spawned, the yolk was seen as a mass of undifferentiated material, occupying all of the egg volume. It had a slightly elliptic shape, rather soft and easily deformed. After a few days, cleavage began reaching the morula stage with > 100 cells within 7 days.

Stage 2. Morula and blastula stages. (8-56 days) The process of segmentation continued, cell became more spherical (morula) and finally reached the blastula stage. This stage was very long and took about 49 days before starting the gastrula stage. There was no space left inside the egg as the yolk took up 100 % of the whole volume of the egg.

Stage 3. Gastrula and germ layers stages. (57-104 days) After the blastula stage, the process of invagination began, a transparent pre-vitelline space was outlined which corresponded to the first differentiated embryonic cells into ectoderm, mesoderm and

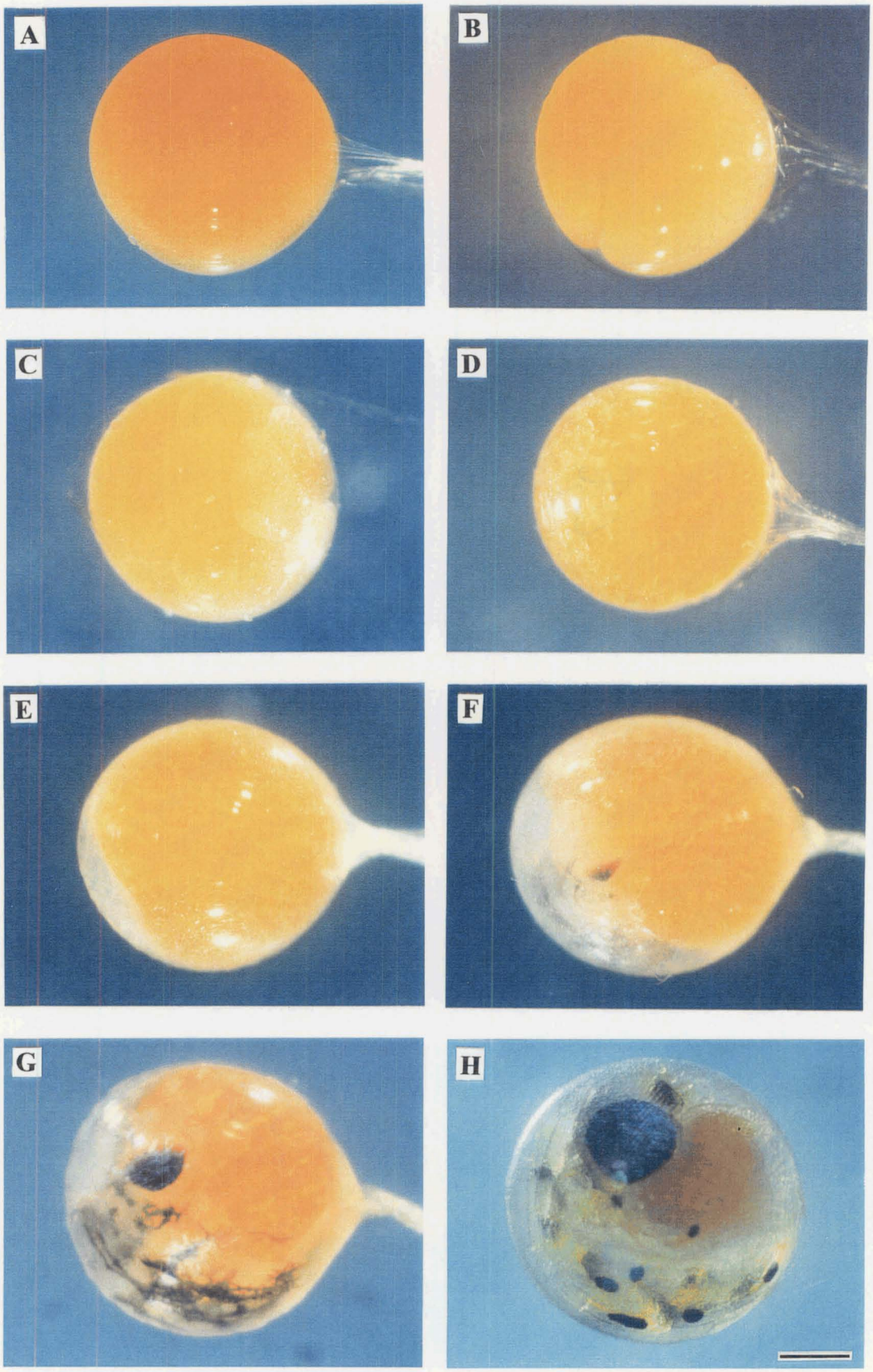


Figure 2.8 Light microscope micrographs of the embryonic development of eggs of *Heterozius rotundifrons*, at 15°C. (A)-(C) stage 1 (D) stage 2 (E) stage 3 (F) stage 4 (G) stage 5A (H) stage 5D. Scale bar = 200 μm.

endoderm. At the end of this stage, the yolk occupied 5/6 of the egg volume.

Stage 4. Eyespots and pigmentation. (105-129 days) At the beginning of this stage, the tissues and organs of the embryos began to be defined. Small dark eye spots were visible as thin crescents, which were the outlines of the eyes. Dark pigments first appeared faintly in the abdominal area as two lines of pigment stripes. At the end of this stage, the yolk occupied 4/5 of the egg volume and the eye index was approximately 92 μm.

Stage 5. Heart-beating. This stage began with the commencement of the heartbeat which became rhythmic towards the end of the stage. During this period, the volume of the egg and the rate of yolk metabolism increased rapidly. This stage can be divided into 4 substages:

Stage 5A. Beginning of heart beat. (130-149 days) This stage started after approximately 130 days of development time when slow heart-beating began. Eyespots and dark chromatophores became more developed. At the end of this stage, the yolk occupied 3/5 of the egg volume and the eye index was approximately 151 μm.

Stage 5B. Yolk in four big lobes. (150-168 days) In the early part of this stage, the yolk started dividing forming four lobes at the end. Also the heartbeat became rhythmic and dark chromatophore pigments including eyespots were well-developed. At the end of this stage, the yolk occupied 1/3 of the egg volume and the eye index was approximately 208 μm.

Stage 5C. Yolk in two big and two small lobes. (169-187 days) Yolk metabolism became very fast and was present as only two lobes at the end of this stage. The heartbeat became faster and the embryo occupied almost the entire volume of the egg. At the end of this stage, the eye index was approximately 264 μm.

Stage 5D Yolk in two lobes (before hatching). (188-194 days) This is the last stage of development, the prehatched embryo developed with a very strong heartbeat. The eye index reached the maximum size of approximately 285 μm.

Embryonic development of Cyclograpsus lavauxi

Compared with *H. rotundifrons*, the eggs of *C. lavauxi* were much smaller but they were similarly slightly ellipsoidal. Newly-laid eggs had an average diameter of 0.262 x 0.288 mm. (i.e. a volume of 0.0104 mm^3 or 10.4 nL). With the progress of development the eggs increased in size and before hatching, they averaged 0.325 x 0.345 mm. (i.e. a volume of 0.0191 mm^3 or 19.1 nL)

The pattern of egg development of *C. lavauxi* was similar to that found in *H. rotundifrons*. However, in this species the incubation period of eggs at 15°C was approximately 56±1 days, approximately one quarter of the period in *H. rotundifrons*. The colour of the egg mass was also different from that of *H. rotundifrons*, it changed from dark purple at the beginning into grey and finally white-grey at the end of the development before hatching occurred. As in *H. rotundifrons*, the embryonic stages of the eggs of *C. lavauxi* can be conveniently defined into 5 stages using the same criteria. However, since the development time of the eggs of this species was rather short, the eggs at stage 5 were divided into 2 substages only. (Table 2.2 and Figure 2.9).

Stage 1. Undeveloped eggs and cleavage stages. (1-4 days) This stage was similar to that found in *H. rotundifrons*, but it took only four days to end the stage.

Stage 2. Morula and blastula stages. (5-11 days) As in *H. rotundifrons*, the eggs at this stage occupied full yolk. In this species, the eggs remained at this stage for approximately 7 days compared with about 49 days for those of *H. rotundifrons*.

Stage 3. Gastrula and germ layers stages. (12-32 days) This stage started with the process of invagination which was similar to *H. rotundifrons*. However, at the end of this stage, the yolk occupied 1/2 of the egg volume compared with 5/6 of *H. rotundifrons*. The total time for development at this stage was about 21 days compared with approximately 48 days for *H. rotundifrons* eggs.

Stage 4. Eyespots and pigmentation. (33-42 days) At this stage, there was a rapid increase in egg volume and the relative quantity of yolk being metabolized in egg at this stage was found to be much greater than in *H. rotundifrons*. At the end of this stage, the yolk occupied 1/4 of the egg volume compared with 4/5 of *H. rotundifrons*. Eye index of eggs at the end of this stage is approximately 52 μm.

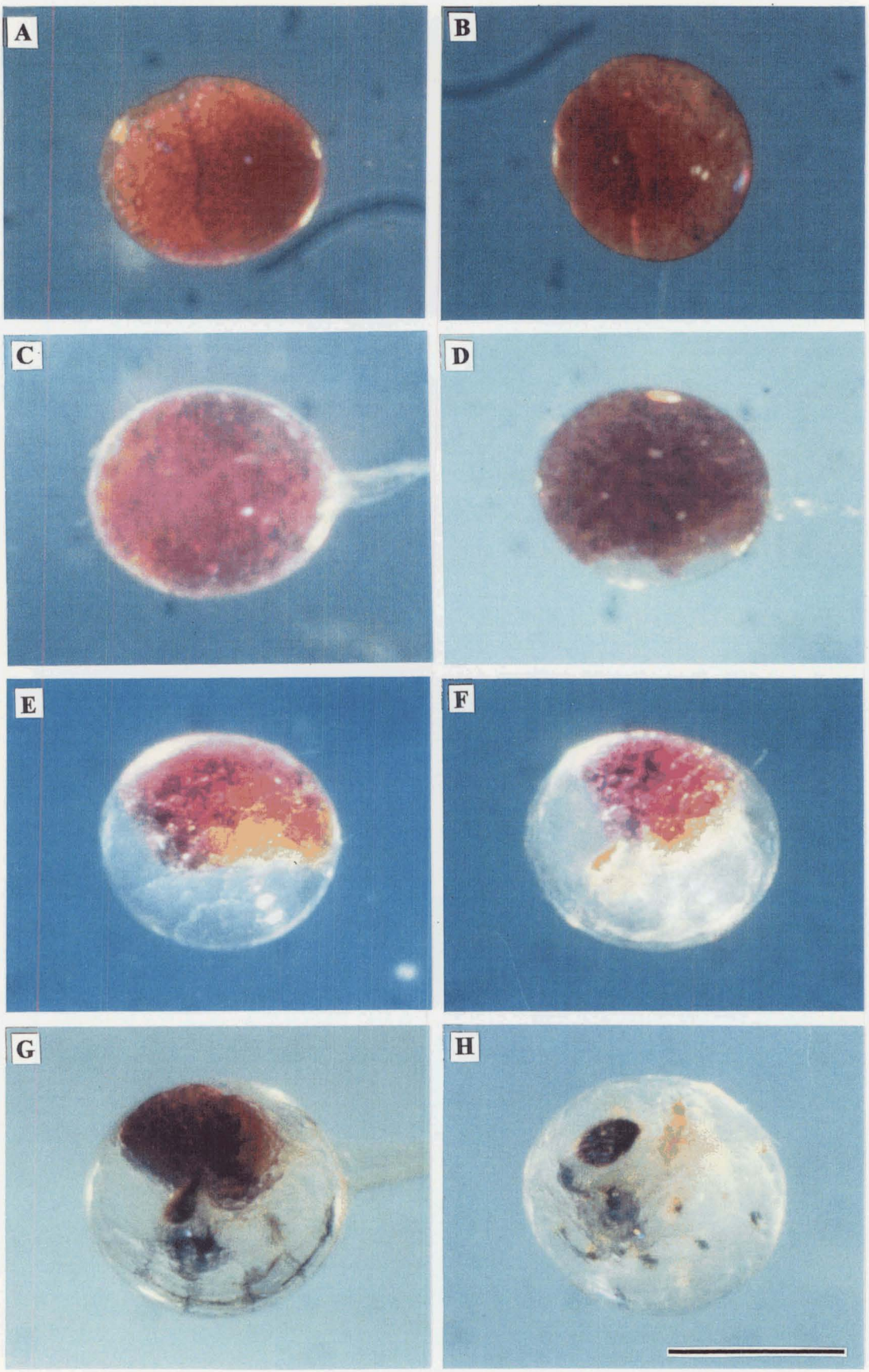


Figure 2.9 Light microscope micrographs of the embryonic development of eggs of *Cyclograpsus lavauxi*,at 15°C. (A)-(B) stage 1 (C) stage 2 (D)-(E) stage 3 (F) stage 4 (G) stage 5A-5C (H) stage 5D. Scale bar = 200 μm.

Stage 5. Heart-beating. As in *H. rotundifrons*, this stage began with the commencement of the heartbeat. As the embryo developed very quickly at this time, only 2 substages are distinguished here:

Stage 5(A-C) Heart-beating. (43-54 days) This stage started after approximately 6 weeks of development time. As in *H. rotundifrons*, slow heart-beating was found at the beginning of this stage. There was no large change in egg volume at this stage in eggs of this species, as observed in *H. rotundifrons*. At the end of this stage, yolk is seen as two lobes and eye index was approximately 80 μm.

Stage 5D Before hatching. (55-56 days) As in *H. rotundifrons*, eggs at this stage occupy two lobes of yolk. The eye index of eggs in this species reached the maximum size of approximately 85 μm.

Internal structure of the egg and its membranes of H. rotundifrons

Unlike telolecithal eggs of most lower vertebrates and some gastropods, crab eggs have yolk concentrated in the interior and the cleavage nucleus is usually at the center of the egg and is surrounded by the mass of yolk spherules and fat droplets ("centrolecithal"; Richards, 1931) (Figure 2.1). Similar features were found in developing eggs of *H. rotundifrons*. The whole volume of undeveloped eggs was occupied by yolk spherules and fat droplets.

According to Richards (1931), cleavage of crab eggs begins by the central-lying nucleus with its surrounding plasmic island multiplying through several nuclear divisions, thus forming a syncytium which no cell boundaries are formed. Cleavage of this type is called "superficial cleavage" (Figure 2.1B-F). The result of superficial cleavage is the formation of a layer of cells around the inner yolk mass. There is no true blastocoele and it is usual to say that the blastocoele is occupied by the yolk mass. In this study, microscopically, the organization of *H. rotundifrons* eggs appeared similar. The eggs began differentiation with yolk cleavage, cell was divided into two cells, four cells and completed as a blastula stage with several hundreds of cells. However, attempts to verify the syncytial nature of eggs at the early stages and the intracellular versus extracellular location of the yolk droplets using T.E.M. were unsuccessful. The high lipid content of the embryos made satisfactory fixation and embedding extremely difficult.

Based on light microscopy, it was found that newly laid eggs (spawned within a few hours) of *H. rotundifrons* had only one single soft membrane (the inner membrane). There was no funiculus and the eggs were easily detached from the pleopods of the female. After 24 hours, the outer membrane and the funiculus (part of the outer membrane) were formed, the latter serving to attach the eggs to the pleopods. Eggs at both the early and late stages of *H. rotundifrons* possessed two membranes, an outer and an inner membrane (Figure 2.10). Unlike the late stage eggs, attempts to resolve the outer membrane from the inner membrane and embryo of eggs at the early stage were unsuccessful. However, the two membranes of eggs at the early stage could be recognized when the eggs were exposed to 125% seawater (Figure 2.10). T.E.M. observation of thin sections of the egg at the early stage also showed that there were two major membranes; the outer and the inner membranes (Figure 2.11). The outer membrane was thicker than the inner one but both consisted of two minor layers. The thickness of the outer and inner membranes were approximately 3.4-4.8 μm and 0.6-0.9 μm respectively. There was a space found between the outer and inner membranes.

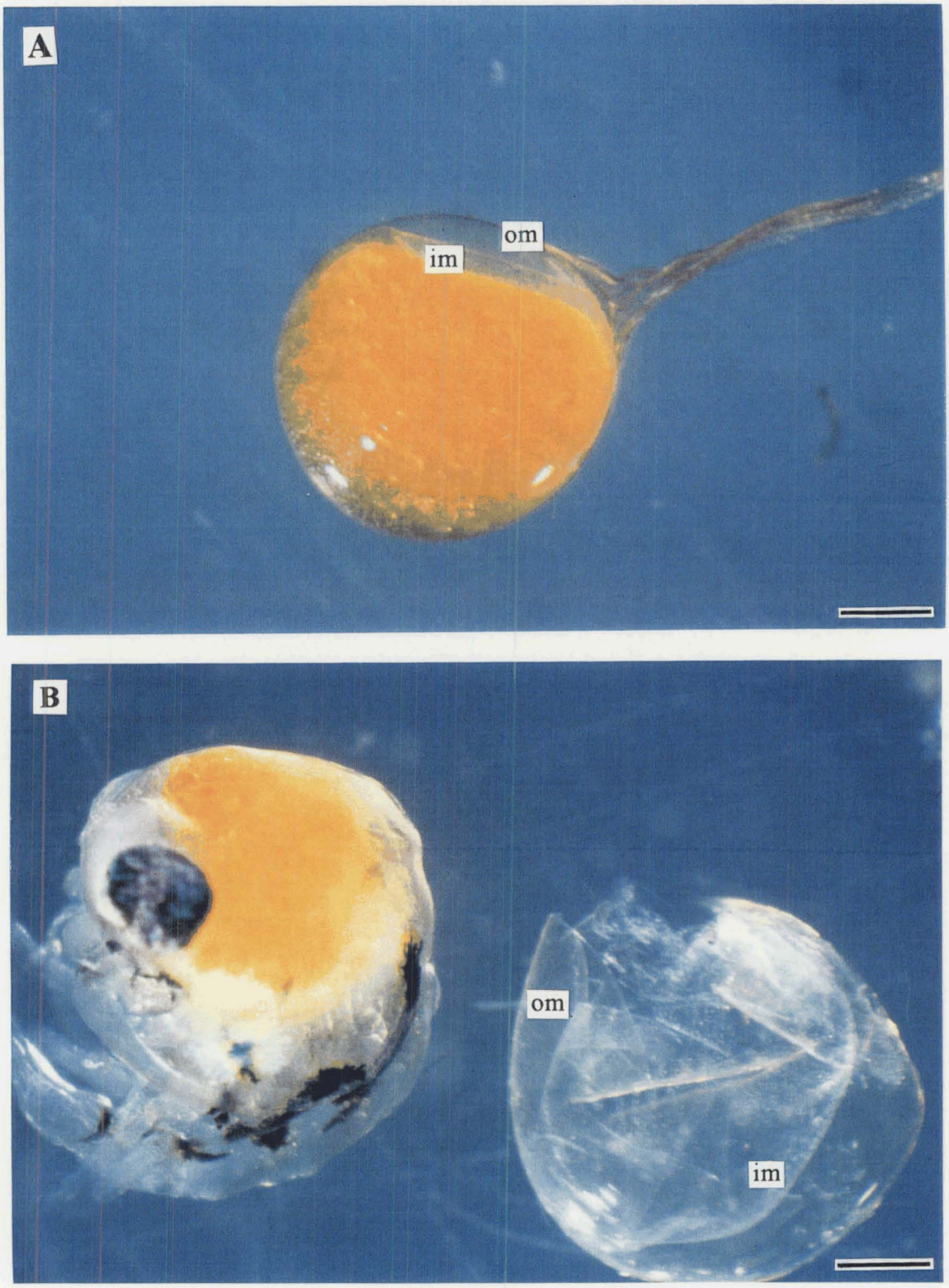


Figure 2.10 Light microscope micrographs of the inner and outer membranes of developing eggs of *Heterozius rotundifrons*. (A) egg at the blastula stage in 125% seawater, showing inner (im) and outer (om) membranes. (B) membranes of a late stage egg dissected off to clearly show the inner (im) and the outer (om) membranes. Scale bar = 200 μm.

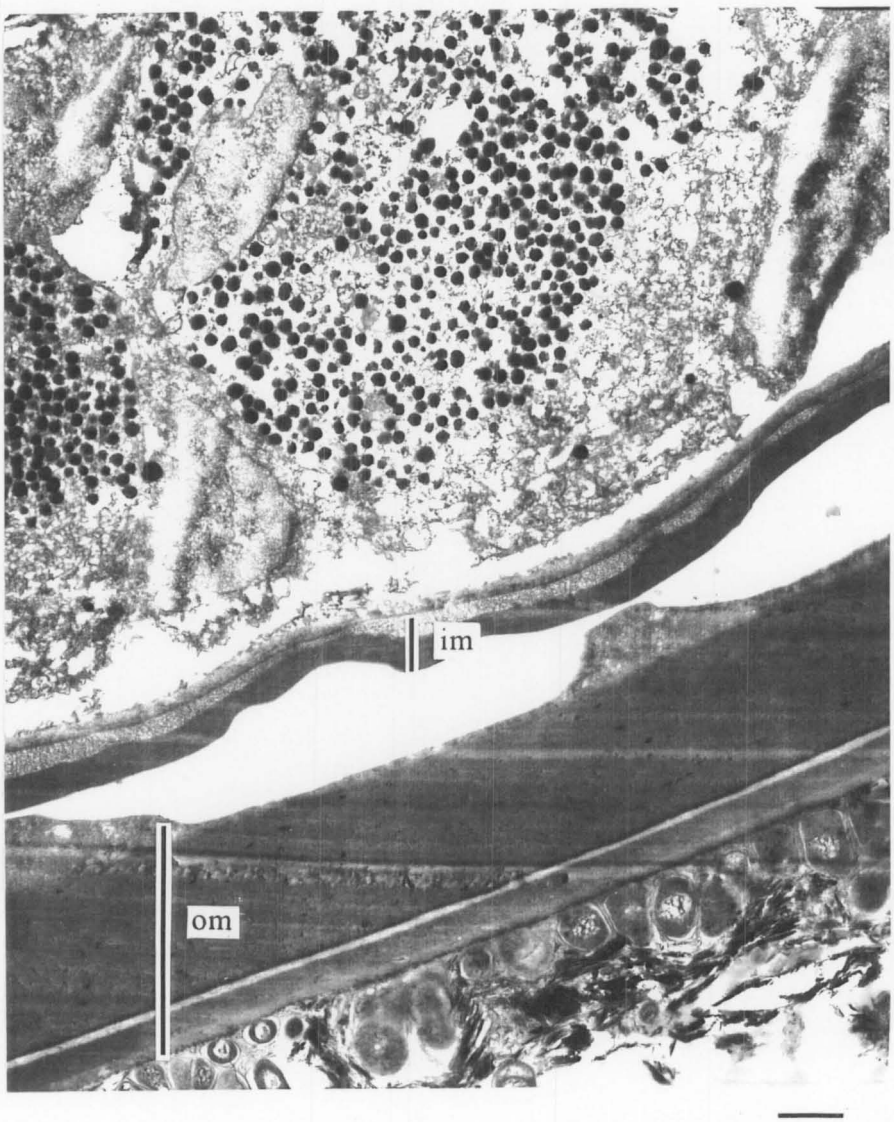


Figure 2.11 T.E.M. micrograph of a developing egg (stage 4) of *Heterozius rotundifrons* showing thin inner (im) and thick outer (om) membranes of the wall. Scale bar = 1 μm.

DISCUSSION

The eggs of *H. rotundifrons* are large and yolky, a feature associated with their slow development (Steele & Steele, 1975). At a constant temperature of 15°C, the incubation period was approximately 194±3 days. From my field observations at Kaikoura, I found that *H. rotundifrons* started spawning around late-March until early-May. By April, most of the eggs were in the blastula stage. This stage lasted until late-June. Between July and August, most of the eggs were in the gastrula stage with yolk about 80% of the whole egg volume. Eyespots and chromatophore pigments became visible in September, and during this period the heart started forming, but there was no sign of beating at this time. In October, most of the eggs were in the heart-beating stage and from this time yolk reduced very rapidly. Eggs at the last stage were found between mid-December and late-January. These data are therefore consistent with a single brood cycle for this species. At Kaikoura, average temperatures ranged from 8°C to 18°C (Rasmussen, 1965), and a somewhat longer incubation period of approximately 8-9 months seems feasible. Branford (1978) reported the incubation periods of *Homarus gammarus* eggs ranged between 3.6 and 9.5 months with the average temperatures between 10.0 and 18.3°C. He indicated that in this species the relationship between incubation period and the average temperatures of a variety of fluctuating temperature regimes was approximately linear. In this study, since, I have never found newly spawned eggs and last stage eggs in other months except the months mentioned above, the incubation period of *H. rotundifrons* eggs is therefore at least 8 months in the field.

Helluy and Beltz (1991) suggested that in invertebrates, the developmental rate of eggs is strongly affected by temperature, however in their studies, they found that *Homarus gammarus* embryos showed a period of arrested development when kept at constant temperature. Perkins (1972) also reported that *Homarus americanus* egg masses kept at seasonal water temperature show a developmental plateau when the temperature is low during the winter months. Since lobster eggs of both species showed periods of arrested development at both constant temperature and seasonal water temperature, Helluy and Beltz (1991) suggested that factors other than temperature have a strong influence on the rate of embryonic development. The embryonic development of 4 of the 21 decapod species studied by Wear (1974) was found to be interrupted by a diapause around the gastrula stage. He found that the diapause periods lasted for between 6 and 16 weeks at temperatures between 11°C and 15°C, and could not be shortened significantly by an increase in temperature. In the present study, it is possible that a diapause period also occurred in *H. rotundifrons* eggs while they were in the stage 3-4 (gastrula and eyespot-

pigmentation stage respectively) at constant 15°C since the eggs showed relatively little change in volume during this time. The "diapause period" refers to a period in which animals suspend their development or growth during the egg, larval, or adult stage (Withers, 1992). Since during the diapause period in *H. rotundifrons*, eggs did not appear to be completely arrested, thus it is more meaningful to refer to the diapause period in eggs of this crab species as a "relative diapause period". The relative diapause period in eggs at the same stages might also occur in natural conditions as these eggs were in stage 3 and 4 during low temperature in winter months.

Wear (1974) reported that in 4 decapod species that showed diapause period, their response to increasing temperature occurred only after the diapause period had been passed. This probably can be used to explain the difference between the incubation period of *H. rotundifrons* eggs in natural condition (at Kaikoura) and at constant 15°C. Since the average temperature at Kaikoura after eggs had passed the diapause period is less than 15°C, the incubation period of eggs in this area should be longer than those period found in constant temperature 15°C.

In *C. lavauxi*, the incubation period of eggs was found to be shorter (56±1 days at constant 15 °C) than those of *H. rotundifrons*. This appears to be related to the smaller size of eggs of *C. lavauxi*. Berrill (1935) found that large tunicate eggs developed more slowly than smaller ones, and McLaren (1966) reported that this also tended to be true for closely related species of copepods. Wear (1974) also suggested that among decapod crustaceans which are closely related, increasing egg size slows down the rate of development. Compared with *H. rotundifrons*, developing eggs of *C. lavauxi* did not show any diapause period, since egg volume of this species increased very rapidly after gastrulation and the incubation period is about the same (approximately 2 months) in natural condition and at constant temperature.

The criteria for characterizing the embryonic stages of both crabs studied here were similar to that used by several authors, such as Pandian (1970a) working with *Homarus gammarus*, Morris (1975) and Amsler & George (1984) with *Callinectes sapidus*. These stages are basically separated by the formation of the blastula, the formation of the gastrula, the appearance of eye spots and pigmentation, and rhythmic heartbeats. In this study, however, the undeveloped and early cleavage eggs were also included as stage I of embryonic stages. Subramonian (1979) who worked with *Emerita asiatica* (H. Milne Edwards, 1837) established 10 stages based on the amount of metabolized yolk and the color of the egg throughout the incubation period. In this study, the amount of yolk left at each stage was also described and used as one of the criteria for

characterizing the embryonic stages.

Newly laid egg or undeveloped egg of *H. rotundifrons* are slightly elliptic shapes and the shapes become more spherical with development. This feature was also found in eggs of *C. lavauxi*, except that the size of eggs of this crab is smaller. The eggs of aquatic invertebrates are thought to increase their volume during embryonic development by a slow but steady osmotic uptake of water (Davies, 1968). Volume of developing eggs of crabs studied here also increased from the time they are first laid to the time of hatching (Table 2.1 and 2.2). The time course of egg volume increase was different in *H. rotundifrons* and *C. lavauxi*. In *H. rotundifrons,* the egg volumes were relatively constant at blastula stage and slightly increased after gastrulation. There was a stronger increase in the volumes of eggs from about the time the embryonic heartbeat was first observable. In *C. lavauxi*, however, this increase occurred after gastrulation.

The quantity of yolk in eggs of both species studied here decreased with the increase of egg volume. However, the amount of yolk left at each stage of egg development was different between two species. In *H. rotundifrons* yolk decreased slowly during the early part of embryonic development and was consumed more rapidly during the last 30% of incubation time. About half of the yolk was left after 75% of incubation time in this species. The relative decrease of yolk during development of eggs of *C. lavauxi* is faster than in *H. rotundifrons*. In this crab, about half of the yolk was left at 57% of the incubation time. Wear (1974) found that in decapod crustaceans, the rate of yolk metabolism varies from species to species according to the time taken for egg development, but in all species the rate was slower during early development than during the last few days before hatching.

"Eye index" was first used to determine the rates and degree of development in lobsters by Perkins (1972). Helluy & Beltz (1991) used Perkins' eye index to stage the embryonic development of the American lobster *Homarus americanus*. In this study, eye index was used as a criteria for staging the embryonic development of both *H. rotundifrons* and *C. lavauxi*. In *H. rotundifrons*, eyespots became visible after approximately 129 days or 66.5% of development time. When first seen, eye index was approximately 92 µm and increased to approximately 285 µm. at the end of development. Eyespots of *C. lavauxi* were seen after approximately 42 days or 75% of development time. In this species, eye index was approximately 52 µm at beginning and became 85 µm at the end of development. Helluy & Beltz (1991) reported that eyespots of *H. americanus* were first seen after the embryo has been developed for 21 days or 13.2% of development time. In this species, the eye index was approximately

70.6 μm when first seen and increased up to 578.2 μm at the end of development. It can be seen that eyespots were formed earlier in *H. americanus* than in two species of crabs studied here. However, it was found that *H. americanus* embryos manifest a period of arrested development after eyespots have been developed, but this period occurred before the forming of eyespots in *H. rotundifrons*.

The studies on the characteristic of egg membrane have been done by many authors in many decapod crustaceans (Ishikawa, 1885; Bumpus, 1891; Herrick, 1891, 1896, 1911; Williamson, 1904; Terao, 1919; Yonge, 1937; Cheung, 1966 and El-Sherief, 1993). In this study, it was found that eggs within the first few hours of spawning, possess only one single membrane or "inner membrane" which is probably secreted by the zygote. The outer membrane was then formed later after eggs has been spawned to the abdomen and this membrane allowed eggs to adhere to the pleopods of female. The observation obtained from this study is similar to those reported by Herrick (1896) and Williamson (1904). Herrick (1896) reported that the ripe egg possesses a single membrane (about 4 μm thickness), which is secreted by the egg follicle and "is unaltered in the course of the passage of the egg through the oviduct." The outer membrane he considered was formed later by the cement glands in the pleopods. Williamson (1904), in his work on *Cancer pagurus*, stated that there are two membranes around the egg, an outermost "chorion" and an inner vitelline membrane. Although he found three layers in sections he regarded the middle one, although thicker than the other two, as being formed by the solidification of the perivitelline fluid. In his opinion this glued the two primary membrane together, thereby ensuring adequate protection for the developing egg. From T.E.M. of this study, it was found that between the two membranes of *H. rotundifrons* egg at the early stage, there is a gap which probably represents the perivitelline space.

In both early and late stages, the inner and outer membranes of *H. rotundifrons* eggs may be seen in the light microscope. Attempts to resolve both membranes from the embryos in eggs at the blastula stage were unsuccessful because they are very closely applied to each other. However the two membranes were easy to recognize after eggs had been exposed to 125% seawater for 24 hours. In eggs at the late stage, the outer membrane can be mechanically separated from the inner membrane, although this was not easy to perform. When trying to separate the inner membrane from the embryo, it was found that one part of the membrane remains attached to the embryo at the caudal region. Yonge (1937) in his work on *Homarus vulgaris*, concluded that the developing eggs are attached, either directly or by way of other eggs. The attachment is by means of strands of transparent "cement" (the funiculus) which also forms the outer membrane of the egg, surrounding an inner membrane which adheres closely to the egg mass. On

chemical and physical tests, he revealed that the outer membrane has properties identical with those of the superficial cuticle of the integument of the female and the inner membrane is chitinous.

A recent study (El-Sherief, 1993) found that incubated eggs of berried female *P. pelagicus* hang from the pleopodal setae by an attaching membrane. He reported that each egg is protected by inner chorion and middle chitinous membrane. The middle chitinous membrane and the outer attaching membrane (has property of phenolic tanning like arthropod cuticle) formed together a distinct case surrounding each egg. This case resists acids and alkalies and appears impermeable to water, salts and dye substances. The chorion of the egg of *P. pelagicus* is free from chitin and consists of the inner lipoid and the outer proteinous layers. Although in this study, I did not analyse the properties of the membranes of eggs of *H. rotundifrons*, I found that the characteristics of this membrane from my observation through microscope were very similar to that of *P. pelagicus* studied by El-Sherief (1993). By observing through the stereo-microscope, the inner membrane was thin and soft, whereas the outer membrane was thick and tough. In my opinion, it is possible that the inner membrane is a chorion secreted by the embryo and being homologous to larval integument since it remains attached to the embryo until hatching. The outer membrane, on the other hand, is secreted by the female since it was formed later after eggs has been spawned. From T.E.M. study, the inner membrane consisted of two minor layers; the inner and the outer which is similar to that reported in *P. pelagicus* (El-Sherief, 1993). In *P. pelagicus*, these inner and outer layers have been tested by histochemical technique and being reported to have properties of lipoid and protein respectively. It is possible that the inner and outer layers of the inner membrane of *H. rotundifrons* also have the same properties as that of *P. pelagicus*. Similarly, from T.E.M. study, the outer membrane of *H. rotundifrons* eggs consisted of two minor layers; the inner and the outer layers. These inner and outer layers probably are corresponding to the middle chitinous membrane and the outer attaching membranes of *Portunus pelagicus* (El-Sherief, 1993) respectively. Thus, it is possible that the inner and outer layers of the outer membrane have properties of chitin and cuticle respectively.

The inner and outer membrane of egg of *H. rotundifrons* probably corresponds to the inner and outer membrane of *Homarus vulgaris* studied by Yonge (1937). Yonge (1937) concluded that the inner and outer membrane have properties of chitin and cuticle respectively which is different from those study by El-Sherief (1993) in *Portunus* eggs. However, Yonge (1937) suggested two functions of the inner membrane: first, to protect the soft eggs as they pass into the abdominal pouch and during attachment; and, second, to provide a firm surface around which the cuticular "cement" can spread and so attach

the eggs to the pleopods. The outer membrane, on the other hand, is the membrane that attaches the eggs to the pleopods. It protects and enables them to withstand the pressure of adjacent eggs and the effect of the constant beating of the pleopods needed to produce the respiratory current around the developing mass of eggs (Yonge, 1946). In addition to the protective function, the inner and outer membranes probably have important functions related to the egg hatching processes. Yonge (1937) proposed that the chitinous membrane may be ruptured by the movements of the embryo, or it may conceivably be dissolved by chitinase in a manner analogous to the dissolution of chitin in the early stages of ecdysis. He also stated that mechanical or chemical rupture of the outer membrane would be more difficult, but the limited permeability of this membrane reveals the possibility of distension and rupture by osmotic pressure. In this study, developing eggs of *H. rotundifrons* also possess two membranes which were similar to those found in *Homarus*. The embryonic development study of these eggs revealed that there was an increase in egg volume during the development. The volume increase is probably brought about by uptake of water. Thus, it is possible that during embryonic development, variations in membrane permeability occur, causing water uptake. This uptake of water along with the increase in egg volume may involve the rupture of the egg membranes and thus the hatching of the embryos. Since eggs possess two membranes, it is thus interesting to know whether there is any difference in their permeabilities. This was investigated in chapter 7.

CHAPTER THREE

HATCHING RHYTHMS AND CONTROL OF LARVAL RELEASE

INTRODUCTION

Crustaceans follow one of two basic developmental patterns: (1) development with a larva hatching at the end of embryonic development; and (2) complete development within the egg which hatches to a juvenile stage (Sastry, 1983). Direct development to the juvenile stage occurs in some Branchiopoda (Cladocera), all Leptostraca, Anaspidacea, Peracarida, and a few Decapoda. Such developmental pattern which extends the embryonic period or eliminates the larvae altogether has evolved in many crustaceans as an adaptation to the environment. Development with a larva hatching at the end of embryonic development is found in most decapod crustaceans, especially in marine species. Typically, decapods with a pelagic larval stage in the life-cycle hatch eggs to release a nauplius or zoea larva.

Timing the hatching of eggs and larval release in many decapod crustaceans has been reported to involve endogenous factors within the egg and female parent, and interaction between endogenous and exogenous factors (Bergin, 1981; Branford, 1978; Christy, 1978, DeCoursey, 1979, 1982; Ennis, 1973; Forward *et al*, 1982; Klapow, 1976; Moller & Branford, 1979; Saigusa and Hidaka, 1978; Saigusa, 1981; Wheeler, 1978; 1975). A coordination of the processes within the egg and the parent, and with the environment may involve physiological and behavioural changes for synchronization of larval release. Endogenous components operating within the egg, female, or both, and exogenous factors synchronizing the hatching rhythm with the onset of darkness, control the rhythm in *Homarus gammarus* (Ennis, 1973) and *H. americanus* (Ennis, 1975). Other decapods also use light for regulating the timing of hatching (Branford, 1978; Forward *et al.*, 1982; Klapow, 1976; MacDiarmid, 1985; Moller & Branford, 1979). Hatching rhythms in which larval release coincides with the night-time high tide have been reported in intertidal crabs *Uca pugilator, U. minax*, and *U. pugnax* (Bergin, 1981; De Coursey, 1979; Salmon *et al.*, 1986); supratidal crab *Sesarma dehaani, S. haematocheir, S. intermedium, S. cinereum* (De Vries and Forward, 1989; Saigusa and Hidaka, 1978; Saigusa, 1981, 1982) and subtidal crab *Rhithropanopeus harrisii* (Forward *et al.*, 1982, 1986). For the subtidal crab *Neopanope sayi*, release was reported to occur near the time of both day and night high tides (De Vries and Forward, 1989). In addition to tidal rhythms, other lunar factors appear to be involved in the semilunar hatching rhythm of the intertidal crabs *Sesarma haematocheir* and *S. intermedium* (Saigusa and Hidaka, 1978).

For warm temperate and tropical brachyurans, egg hatching is a short duration event, with all eggs present on a female usually hatching within 15-30 min (De Coursey, 1979; Forward *et al.*, 1982). Exceptions occur especially among the Xanthidae which occasionally release a group of larvae each night for two or three consecutive nights (Forward *et al.*, 1982; Christy, 1986; De Vries and Forward, 1989). Similarly, in lobster (Ennis, 1973; Branford, 1978; Moller and Branford, 1979), larvae hatch at the same time each night over a period as long as 6-7 weeks. The fiddler crab *Uca* releases larvae for several minutes, in synchrony with nocturnal high water (DeCoursey, 1979, 1983). Such a short and precisely timed event implies that synchronous hatching within each batch of an egg mass must be the result of synchronous development of the embryos in that batch and factors which control the precise time of hatching (De Vries and Forward, 1991). This raises the question of what is the factor controlling the timing of hatching? If hatching is under endogenous control then the timing may be controlled by the female, the developing embryos or both. Forward (1987) suggested that rhythms in larval release are controlled by endogenous factors. He concluded that the site of endogenous control among brachyurans probably varies with adult habitat. For species living in high intertidal and supratidal areas, the "clock" may reside in the female (DeCoursey, 1979; Saigusa, 1981) while in the subtidal it resides in either the female or the developing embryos depending upon the species (Pandian, 1970b; Ennis, 1973; Forward and Lohmann, 1983).

Few data exist on larval release rhythm in New Zealand decapods. The only study concerning the larval release rhythm in New Zealand decapods was done in rock lobster *Jasus edwardsii*. In this subtidal species, the release was found to be related to light-dark cycle, with the release mostly occurring at the beginning of the light phase (MacDiarmid, 1985). New Zealand intertidal crabs *Heterozius rotundifrons* (Belliidae) and *Cyclograpsus lavauxi* (Grapsidae) have been previously studied on many aspects of both physiology and ecology (Leader & Bedford, 1978; Jones, 1978; Shanks, 1982; Pellegrino, 1984; Innes *et al.*, 1986; Waldron *et al.*, 1986; Dewa, 1990; Field, 1990; Snell, 1991; Olsen, 1992), but no previous data on the ecophysiological adaptations of developing eggs and ovigerous females including the timing of larval release have been reported (responses to air exposure and salinity changes on developing eggs and ovigerous females are considered in later chapters). Both *H. rotundifrons* and *C. lavauxi* are found inhabiting the intertidal zone along the rocky shore where they are exposed to highly variable environmental conditions. Air exposure and salinity change during the period of low tide are potential problems for hatching crab larvae (zoea). Zoea larvae appear unequipped for aerial exposure. Therefore, larval release in these crabs is expected to be related to the tidal cycle or period of immersion on the shore.

Differences in position on the shore between these two species (*C. lavauxi* and *H. rotundifrons* are high and low shore crabs respectively) might thus affect the pattern of larval release. As noted above, the site of endogenous control of larval release among brachyurans varies with adult habitat (Forward, 1987). Additionally, the duration of development of eggs in both species (approximately 2 and 6 months at 15°C in *C. lavauxi* and *H. rotundifrons* respectively) might also influence the time of larval release. The variation in development stage of eggs within the same batch is likely to be greater with longer total development times. Thus, achieving synchrony in a single burst of hatching is more difficult. The primary objective of this study was therefore to document external factors associated with larval release in *H. rotundifrons* and *C. lavauxi*. It is of interest to know whether hatching in both species occurs rhythmically with light-dark and tidal cycles. Do these crabs show patterns in larval release similar or different to other intertidal crabs?

In this study, ovigerous crabs of both *H. rotundifrons* and *C. lavauxi* with prehatching eggs were entrained to experimental tidal and light/dark (LD) cycles. The timing of larval release was then examined during several days of continuous immersion (and continuing light/dark cycle). It will be shown that for *C. lavauxi*, larval release is a single event, is independent of the light cycle and synchronised with the previously entrained tidal cycle. *H. rotundifrons*, by contrast, releases larvae in several bursts which appear to be related to both the time of day and the tide. The demonstration of release rhythms in the absence of a direct tidal cue raises the question of their control. Is the tidal rhythm entrained in the eggs themselves or in the adult female crab? What event initiates hatching and, once started, how is it synchronised within the batch? In some subtidal species it has been shown that release of a water-borne pheromone by hatching larvae induces abdominal pumping by the female which presumably assists mass-hatching (Forward *et al.*, 1987). A complete analysis of factors controlling the timing of hatching was beyond the scope of this study. However, initial experiments comparing the hatching of eggs detached and isolated from the female with those still attached, provide a little further information for the two species studied here.

MATERIALS AND METHODS

Collection and maintenance of Ovigerous Crabs

Preliminary observations indicated that in their natural habitat, the period of larval release in both *H. rotundifrons* and *C. lavauxi* was during summer time, between December and January. Ovigerous females of both species were collected from the intertidal zone at Kaikoura. Approximately 100 ovigerous crabs, *H. rotundifrons,* were collected in December 1993 and 1995 during the period in which their eggs were in the last stage of development. Others were collected in April 1993 and 1995 during the period of their spawning. Approximately 100 ovigerous females of *C. lavauxi* were collected only in December 1993 and 1995. *C. lavauxi* were collected from under boulders at high tide level, whereas *H. rotundifrons* were found half buried in sand and gravel, under stones and pebbles lower on the shore. Crabs were transported to the University of Canterbury in buckets containing seawater and maintained in a recirculating tidal tank system (Figure 2.3, in chapter 2). The tidal systems provided alternate periods of immersion (high tide) and emersion (low tide) of equal duration (6h 12 min). Thus, high and low tide times in this system are defined as time at the mid points of immersion and emersion phase respectively. The tidal tank system was set up in a controlled temperature room at 15°C under a 12h light : 12h dark cycle (12hL:12hD) with the light on at 7.00 a.m. and the light off at 7.00 p.m (NZDT). Crabs were fed with chopped mussel tissue once a week. The seawater was changed every week and salinity was maintained between 33 and 35 ppt throughout the study period.

Rhythm of Larval Release in H. rotundifrons and C. lavauxi

In an earlier study using *Rhithropanopeus harrisii*, 5 days is a sufficient time to reset its biological clock (Forward, *et al.*, 1982). Therefore in my experiments I acclimated ovigerous crabs to the new environmental cycle in laboratory for a period of at least one week prior to recording larval release. Any crabs that released larvae within 7 days of collection were discarded. Many of the crabs were maintained in these laboratory conditions for several months prior to spawning. Crabs were checked every few days for the developmental stage of their embryos and females with embryos determined to be prehatch were then removed for experiments.

To determine the time of larval release, crabs with embryos were placed individually into hatching bowls consisting of two plastic cups: the upper cup (5 cm high and 7 cm diameter) with mesh of 0.2 mm in the bottom fitted into a lower cup (7 cm high and 7 cm diameter) containing seawater at the volume of approximately 300 mL. The mesh in the top cup permitted hatching larvae to swim down into the bottom cup. This hatching cup allowed easy transfer of the female without direct handling. Experiments were done in the same controlled temperature room as the tide-tank. Seawater in each crab's bowl was changed daily by replacing the lower cup. Crabs that were expected to release their larvae on a given day (based on microscopical observation of a few eggs) were observed every hour during the period between 5.00 p.m. - 7.00 a.m. (with the light on at 7.00 a.m. and the light off at 7.00 p.m.) while the eggs were hatching. The observations were continued, and the female was transferred to a new bowl until all larvae were released. Most crabs commenced release within 1-2 days. Crabs which commenced release after 3 days were discarded. The number of larvae released within each 1 hour interval and the time of larval release were then recorded. A torch covered with a sheet of red cellophane was used to see the larvae during the dark phase. For *H. rotundifrons*, all larvae were counted, but for *C. lavauxi*, the sample of seawater with zoea larvae was mixed up and one subsample (either 1, 5 or 10 mL) was taken. The number of larvae of the subsample was counted and the total number of larvae was then estimated.

It was impossible to record hatching continuously for the full 24 hours period on consecutive days. Preliminary observations indicated that female crabs of *H. rotundifrons* released larvae mostly at night, whereas *C. lavauxi* released larvae both at day and night time. Thus, initial experiments compared larval release of *H. rotundifrons* and *C. lavauxi* during an "intensive sampling period" commencing two hours before the end of light phase and continuing until the end of dark phase. Eggs which hatched at times other than during the intensive sampling period were designated as having hatched at "other times". For *C. lavauxi*, a subsequent experiment was set up with the intensive sampling period in the day time between 9.00 a.m. and 5.00 p.m. (with the light on at 7.00 a.m. and the light off at 7.00 p.m.). Observations on larval release were made at one hour intervals during this period. The recording on larvae release of *C. lavauxi* was completed within a single intensive sampling period as crabs invariably released a whole clutch of larvae within a few hours. However, in *H. rotundifrons*, as one crab released larvae over 4-6 consecutive nights, the recordings were made for 4-6 successive sampling intervals or until no more eggs remained. The mean release times for a particular day for an individual of either species was calculated by weighting the time intervals by the number of larvae released in each interval. The relationship between

larval release and the tidal cycle was examined in plots of mean release time for consecutive night-time or day-time tides. The slope of these lines, the incremental release lag (h/tide) were obtained by linear regression and were compared with the tidal lag (0.4 h/tide, set by the artificial tide system) using a single-sample t-test (Solo, BMPD Statistical Software, Inc.).

Hatching of Detached Eggs of H. rotundifrons and C. lavauxi

To investigate whether the timing of hatching of *H. rotundifrons* and *C. lavauxi* was initiated by the embryos themselves or by the female crabs, hatching of the detached eggs was compared with the attached eggs. Crabs with eggs just about to hatch were used for the experiments. A sample of eggs (approximately half of the egg mass) was removed from the female and placed in a small petri-dish containing pasteurized seawater. Meanwhile the same female was placed in a hatching bowl. For *H. rotundifrons*, experiments were done at the intensive sampling period of the dark phase throughout the hatching period. Larvae were counted using the method mentioned previously. The hatched larvae were identified and recorded as swimming and non-swimming larvae. For *C. lavauxi*, recordings were made at the intensive sampling period of the light phase. Larvae were counted using the same method. Due to the large number of small eggs, it was difficult to estimate the number unhatched at the end of experiments. Thus, in this species, detached eggs were estimated by subsampling at the beginning and the number of unhatched eggs at the end estimated by difference. In this species, only the swimming larvae were recorded as hatched. All experiments were performed in the controlled temperature room at 15°C, under 12hL:12hD.

RESULTS

Rhythm of larval release in H. rotundifrons

Figure 3.1 shows pattern of larval release by three individual *H. rotundifrons*. It can be seen that each crab has a similar pattern in which larval release occurred for about 4-6 consecutive nights. Timing for the larval release of these crabs was close to the time of the immersion period at night and the number of released larvae increased from the first night to the peak at about day 3 of hatching period. Mean ± SE of the total number of larvae hatched per one crab during the "intensive sampling period" (12h dark + 2h light) and the "other time" (10h light) of 57 ovigerous *H. rotundifrons* under a 12hL:12hD cycle were compared in Table 3.1. Less than 1 % of larvae were released during the light phase. The distribution of hatching time for larval release of these crabs are shown in Figure. 3.2. When the immersion period occurred before or around the beginning of the dark phase, most hatching occurred a few hours after the beginning of immersion time. When the immersion phase occurred after a few hours of the dark phase, hatching occurred around the immersion time. Thereafter hatching was advanced as the immersion time occurred later in the dark phase. On days when immersion period occurred twice, near the beginning and around the end of dark phase, most hatching occurred at the early part of dark phase. Regressions were determined for the relationship between the mean time of release (clock h) and total elapsed time (number of tides) (Figure 3.3). Using all of the data points (tides 3-29) the slopes of the regression was 0.244±0.41 h.tide^{-1}. This line is significantly different from zero (t-test; $P < 0.001$) expected if there is no relationship between the tide and hatching, and is also significantly lower than the value 0.4 ($P < 0.001$) expected if release is tightly linked to the tidal cycle. Examination of the mean data for each tide (Figure 3.4) suggests that the incremental delay is not constant and appears to be divided into two periods. Initially (tides 3-19), hatching was delayed by 0.185±0.021 h.tide^{-1} (significantly different from 0.0 and 0.4 $P < 0.001$). Later, as the hatching time and immersion coincided (tides 19-29) hatching was delayed at 0.355±0.046 h.tide^{-1}, approximating the tidal increment (significantly different from zero, $P < 0.001$; not significantly different from 0.4 $P > 0.3$).

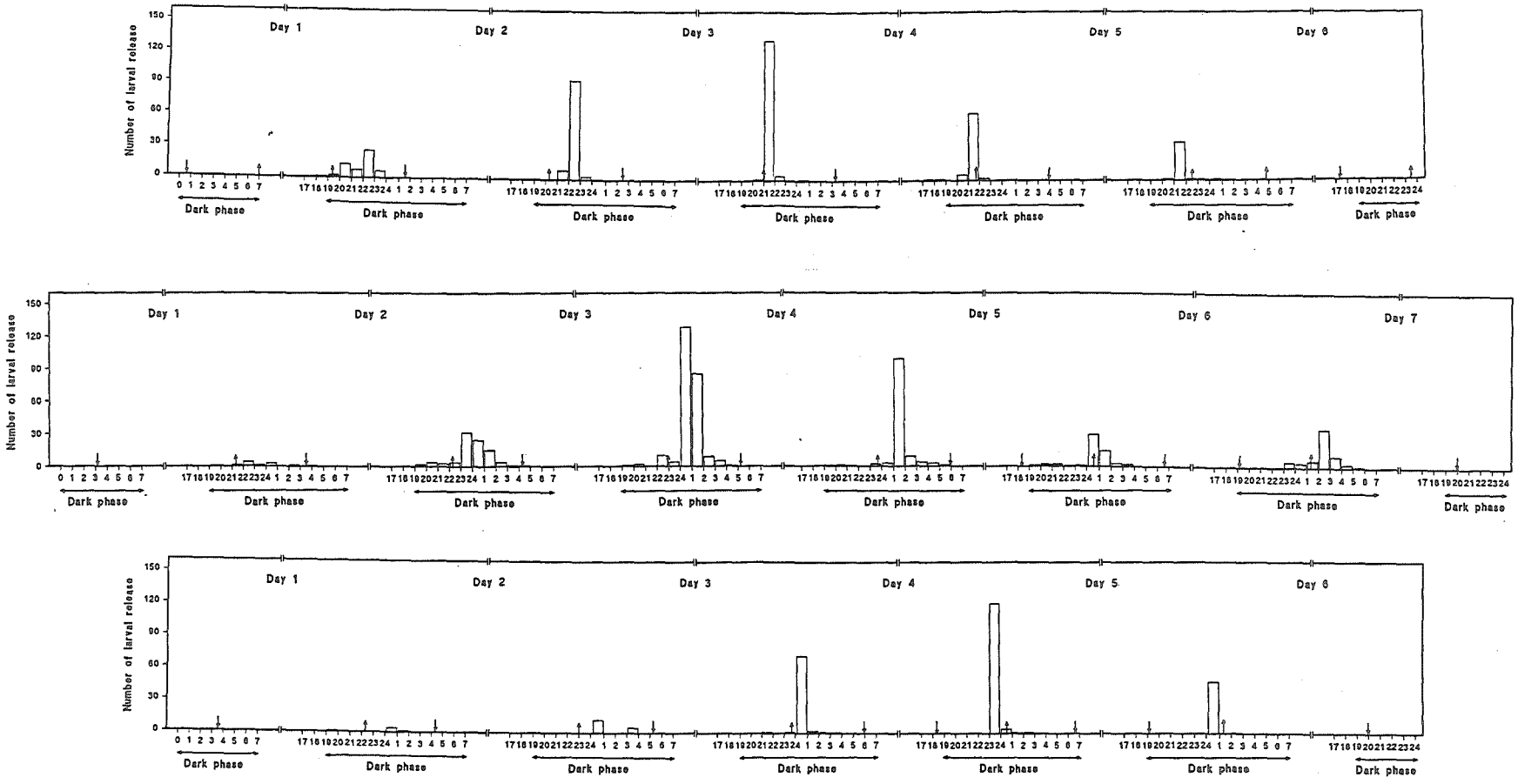


Figure 3.1 Pattern of larval release of *Heterozius rotundifrons* under 12hL:12hD cycle. Crabs release larvae for 5-6 consecutive nights during high tide period. The upward and downward solid arrows represent the commencement time of immersion and emersion respectively corresponding to the previously entrained tidal cycle (crabs were continuously immersed during these observations).

Table 3.1 Comparison of mean ± SE of the total number of larvae hatched per crab during the "intensive sampling period" (12h dark + 2h light, 5.00 p.m. - 7.00 a.m.) and the "other time" (10h light, 7.00 a.m. - 5.00 p.m.) of 57 ovigerous *Heterozius rotundifrons* under 12hL:12hD cycle (with the light on at 7.00 a.m. and the light off at 7.00 p.m.). Time period of observation was 4-6 days for each crab.

Crab No	Total number of larvae hatched during the "intensive sampling period"	Total number of larvae hatched during the "other time"	Crab No	Total number of larvae hatched during the "intensive sampling period"	Total number of larvae hatched during the "other time"
1	185	0	30	470	7
2	464	0	31	167	4
3	584	1	32	413	0
4	353	6	33	227	0
5	271	0	34	405	1
6	257	0	35	227	3
7	282	0	36	276	7
8	177	3	37	317	3
9	395	12	38	290	11
10	393	7	39	378	1
11	334	2	40	197	0
12	289	0	41	256	9
13	360	2	42	224	1
14	396	4	43	285	3
15	435	0	44	175	0
16	353	0	45	256	1
17	368	5	46	288	7
18	271	0	47	170	1
19	436	0	48	325	4
20	451	1	49	209	0
21	448	13	50	190	3
22	381	5	51	317	2
23	432	0	52	270	0
24	253	0	53	255	5
25	483	2	54	260	0
26	467	6	55	347	9
27	541	4	56	259	4
28	404	1	57	253	3
29	419	0	Mean ± SE	326 ± 13	2.85 ± 0.44

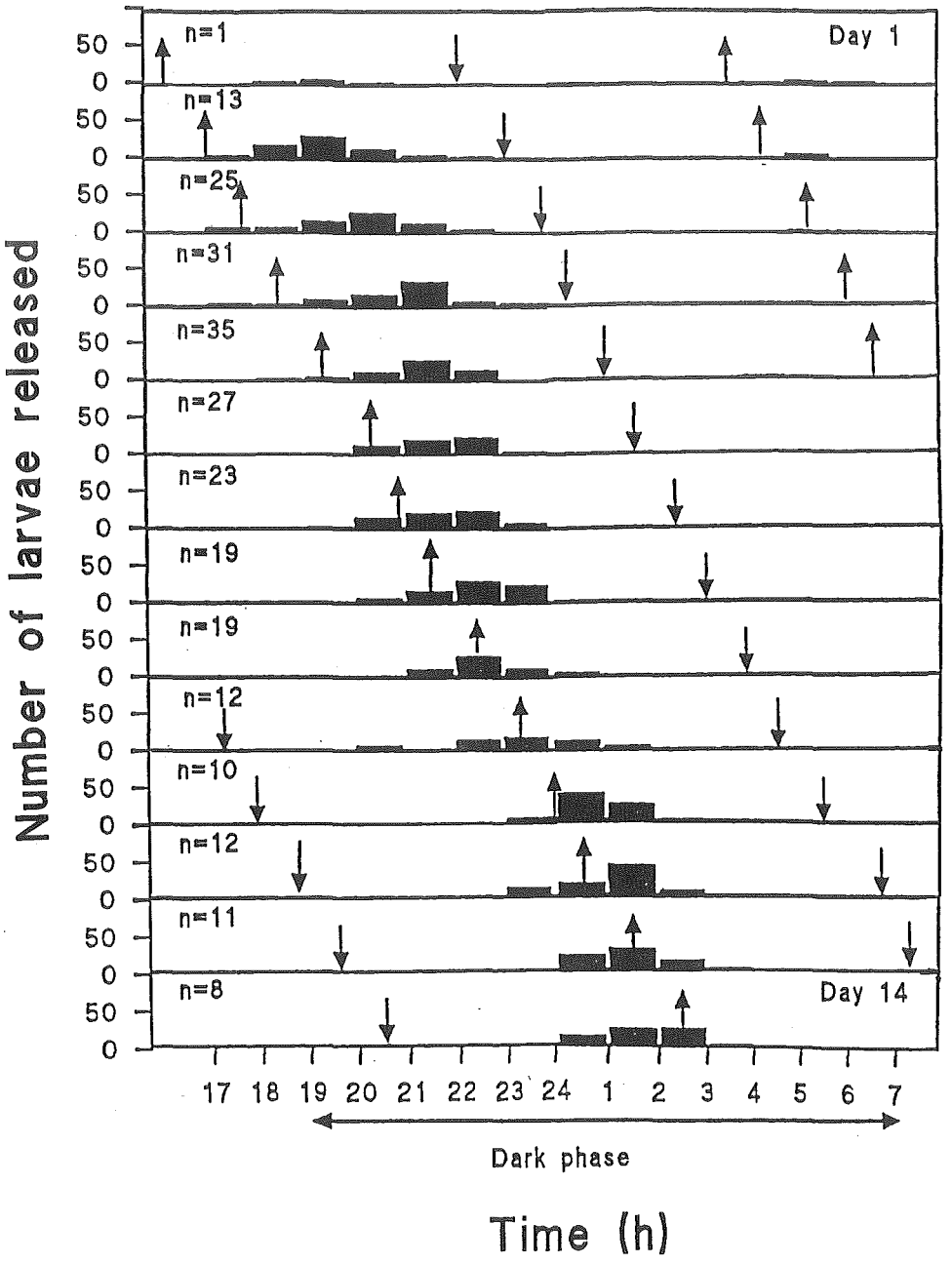


Figure 3.2 Distribution of mean times of the average number of larval release per ovigerous *Heterozius rotundifrons* (total number of larvae released is divided by n, the number of crabs releasing larvae) under 12hL:12hD cycle. Crabs were acclimated to a new tidal cycle for a period of at least one week before larvae had been released. The upward and downward solid arrows represent the commencement time of immersion and emersion respectively corresponding to the previously entrained tidal cycle (crabs were continuously immersed during these observations). Hatching observations were made under conditions of continuous immersion. (Number of crabs = 57, crab release days = 246).

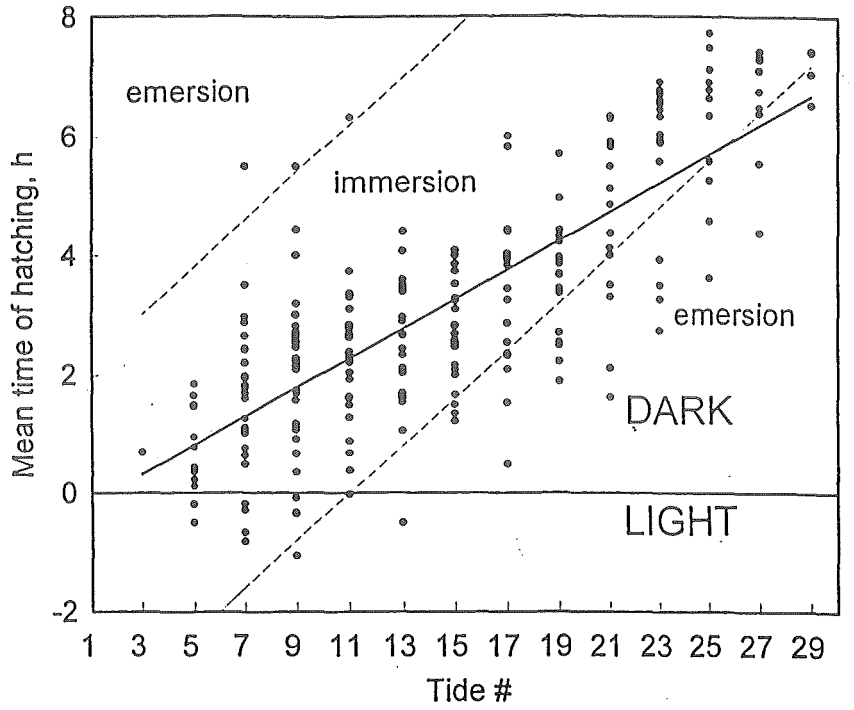


Figure 3.3 *Heterozius rotundifrons*. Regression of mean release times on consecutive high tides (at night) for data in Figure 3.2. Dashed line represents the start and end of immersion periods corresponding to the previously entrained tidal cycle. Time zero represents start of dark phase. Each point (n = 237) represents the mean hatching time of a single crab (several hundred larvae released over 3-5 h). The line represents the regression y = 0.2444(x) - 0.4138.

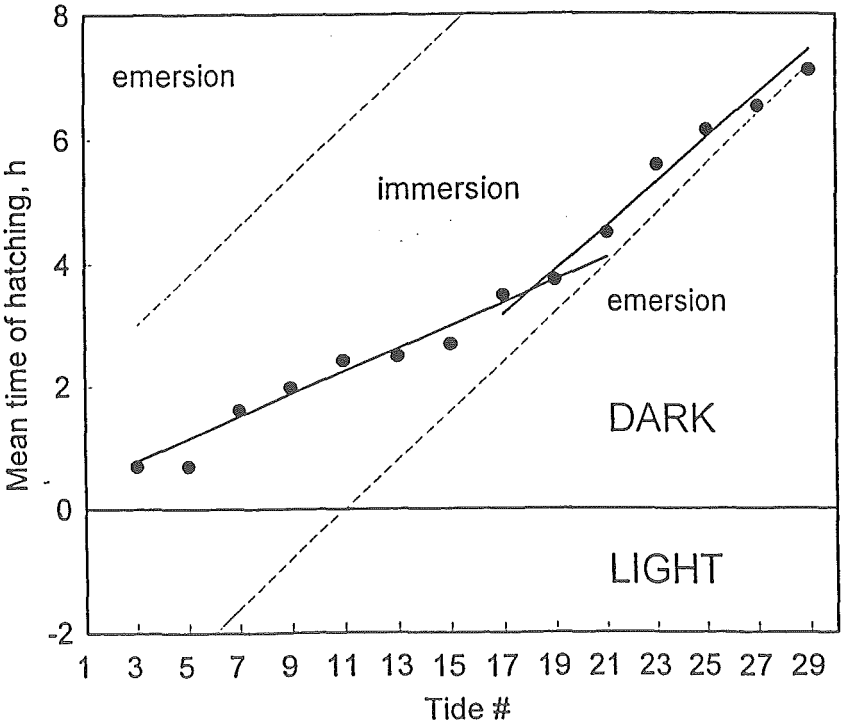


Figure 3.4 *Heterozius rotundifrons*. Same data as in Figure 3.3, but regression lines are plotted separately for the release on tides 3-19 and on tides 19-29. Points are the mean values for all crabs by regressions carried out on individual data. The lines represent the regression y = 0.185(x) + 0.2122 and y = 0.355(x) - 2.8686 for the release on tides 3-19 and on tides 19-29 respectively.

Rhythm of larval release in C. lavauxi

Unlike *H. rotundifrons*, *C. lavauxi* released larvae in only one burst . A whole clutch of larvae was released within a few hours. The total number of larvae hatched per crab during the dark phase, light phase and light-dark phase of *C. lavauxi* under a 12hL:12hD cycle were compared in Table 3.2. The distribution of hatching time for these crabs are shown in Figure. 3.5. Figure 3.6 shows the distribution of hatching time of crabs which released larvae during the intensive sampling period in the day time (between 9.00 a.m. and 5.00 p.m.). Larval release of this species occurred during the high tide period regardless of whether it was day or night. Regressions were carried out to determine the incremental delay of hatching time on consecutive tides (Figure 3.7-3.9). The slopes of the regression lines for releasing at both night and day time were significantly different from zero (t-test; $P < 0.001$). The mean and SE of the slope of the regression line for release at night was 0.335±0.024 h.tide^{-1} which is significantly different from 0.4 h.tide^{-1} of the mean immersion time ($P < 0.01$). The slope of the regression line of the time release at day was 0.272±0.068 h.tide^{-1} which is not significantly different from 0.4 h.tide^{-1} ($P > 0.06$). When the night and day data were combined, a slope of 0.402±0.013 h.tide^{-1} was obtained which is not significantly different from 0.4 h.tide^{-1} ($P > 0.8$).

Hatching of detached eggs of H. rotundifrons and C. lavauxi

Both *H. rotundifrons* and *C. lavauxi*, detached eggs were able to hatch into larvae independently of the female. Detached eggs of *H. rotundifrons* continued to hatch during the dark phase of the period of observation (3-5 days) as in attached eggs. At the end of hatching period, a number of detached eggs were left as unhatched eggs. Some unhatched eggs were still alive, but some had died. About a half of hatched larvae of detached eggs were healthy and could swim. Mean ± SE of percentage of detached eggs hatched as swimming and non-swimming larvae are 16.52% and 22.22% of the total number of detached eggs respectively (Table 3.3).

Compared with *H. rotundifrons*, the percentage of detached eggs hatched at the same period as attached eggs of *C. lavauxi* was much lower (about 3.44%). However, hatching of detached eggs continued throughout both low and high tide periods following the hatching of attached eggs and the overall production of swimming larvae was higher than in *H. rotundifrons* (after 24 h 23.29%, Table 3.4).

Table 3.2 Total number of larvae hatched per crab and period of hatching (L/D = hatching occurred between the light-dark period; L = hatching occurred during the light; D = hatching occurred during the dark) of *Cyclograpsus lavauxi* under 12hL:12hD cycle (with the light on at 7.00 a.m. and the light off at 7.00 p.m.).

Crab No.	Total number of hatched larvae	Period of hatching	Crab No	Total number of hatched larvae	Period of hatching
1	7594	L/D	28	3408	L
2	5304	L/D	29	7024	L
3	21594	L/D	30	9086	D
4	6123	L/D	31	6792	L
5	5280	L/D	32	8315	L
6	6970	L	33	9256	L
7	9766	L/D	34	5397	L/D
8	6390	L	35	7737	D
9	15336	L	36	6346	D
10	5833	L/D	37	12142	L
11	7982	D	38	9670	D
12	6968	D	39	5474	D
13	3574	L	40	8843	D
14	12397	D	41	6321	D
15	9936	L	42	7385	D
16	5980	L	43	6610	D
17	5560	L/D	44	6470	L
18	10154	D	45	9892	L
19	4485	D	46	6551	D
20	9484	L	47	6227	D
21	15064	L	48	7539	D
22	6104	D	49	5628	L/D
23	8480	L	50	3570	L/D
24	5483	D	51	9579	L/D
25	15220	L	52	10800	L/D
26	6710	D	53	4578	L/D
27	10704	D			

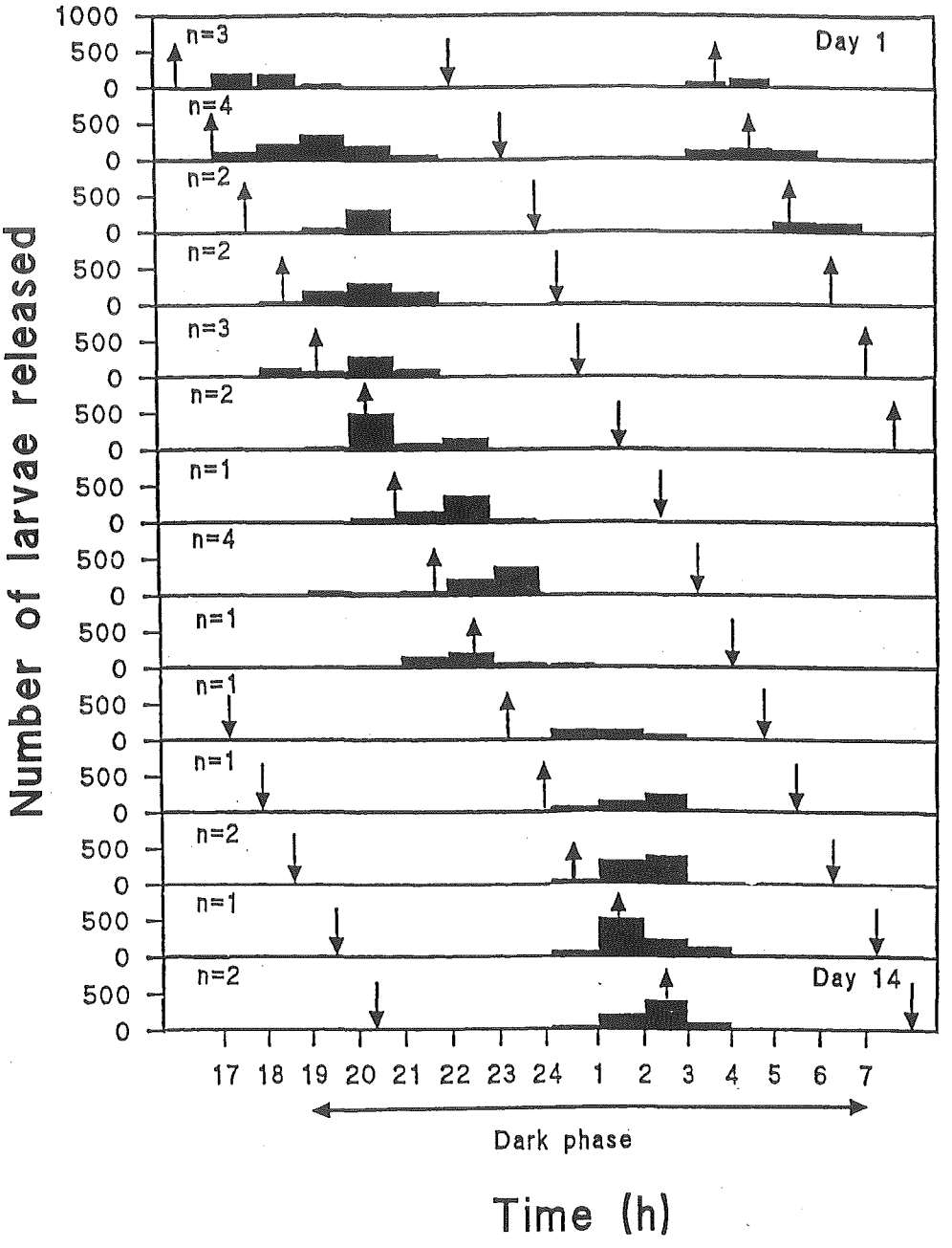


Figure 3.5 Distribution of mean times of the average number of larval release per ovigerous *Cyclograpsus lavauxi* (total number of larvae released is divided by ten and by n, the number of crabs releasing larvae) under 12hL:12hD cycle. Crabs were acclimated to a new tidal cycle for a period of at least one week before larvae had been released. The upward and downward solid arrows represent the commencement time of immersion and emersion respectively corresponding to the previously entrained tidal cycle (crabs were continuously immersed during these observations). Hatching observations were made under conditions of continuous immersion. (Number of crabs = 29, crab release days = 29).

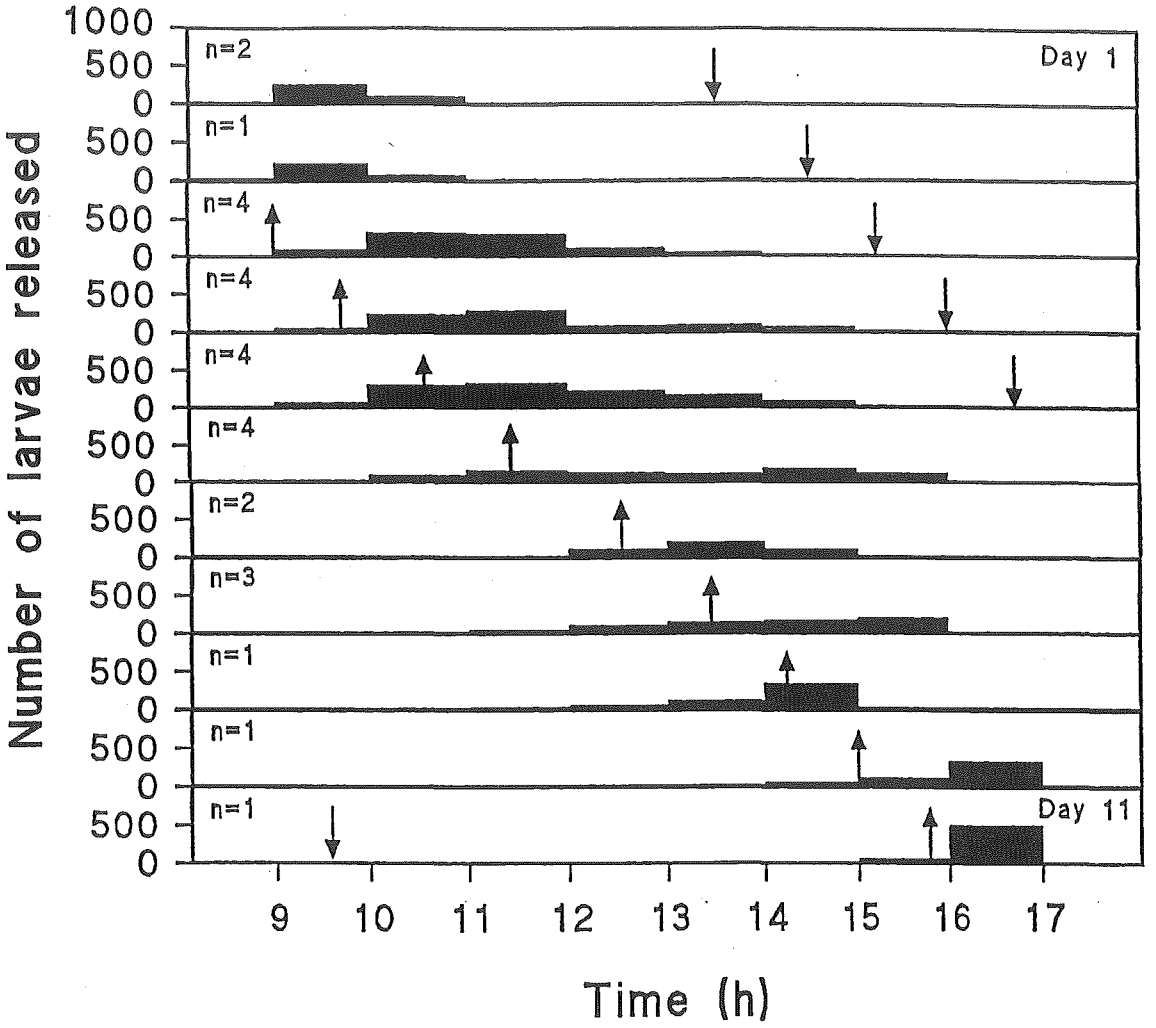


Figure 3.6 Distribution of mean times of the average number of larval release per ovigerous *Cyclograpsus lavauxi* (total number of larvae released divided by ten and by n, the number of crabs releasing larvae) under 12hL:12hD cycle. Crabs were acclimated to a new tidal cycle for a period of at least one week before larvae had been released. All recordings were done between 9.00 a.m. to 5.00 p.m. The upward and downward solid arrows represent the commencement time of immersion and emersion respectively corresponding to the previously entrained tidal cycle. Hatching observations were made under conditions of continuous immersion. (Number of crabs = 27, crab release days = 27).

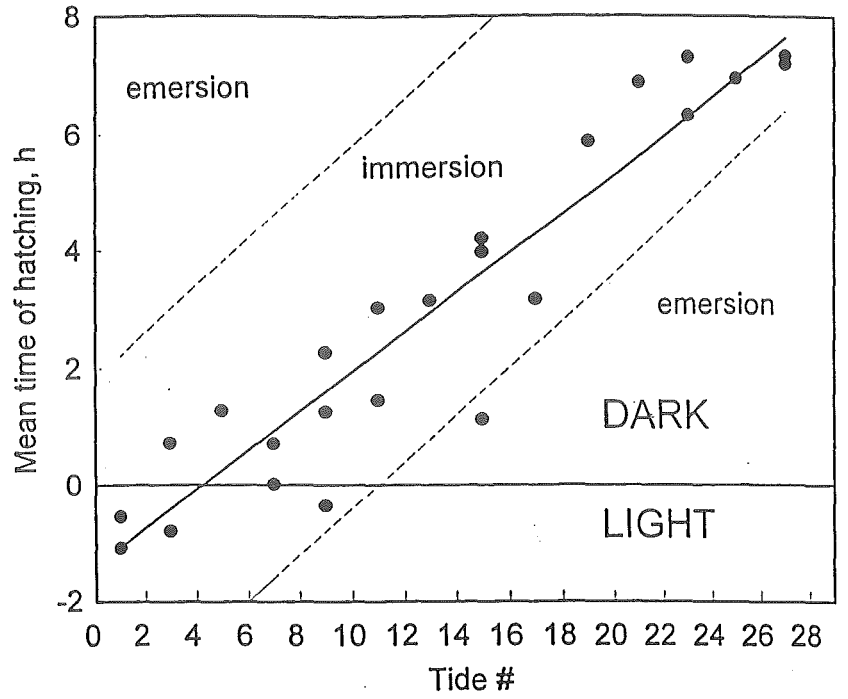


Figure 3.7 *Cyclograpsus lavauxi*. Regression of mean release times on consecutive high tides (at night) for data in Figure 3.5. Dashed line represents the start and end of immersion periods corresponding to the previously entrained tidal cycle. Time zero represents start of dark phase. Each point (n = 24) represents the mean hatching time of a single crab (several thousand larvae released over 3-5 h). The line represents the regression y = 0.335(x) - 1.417.

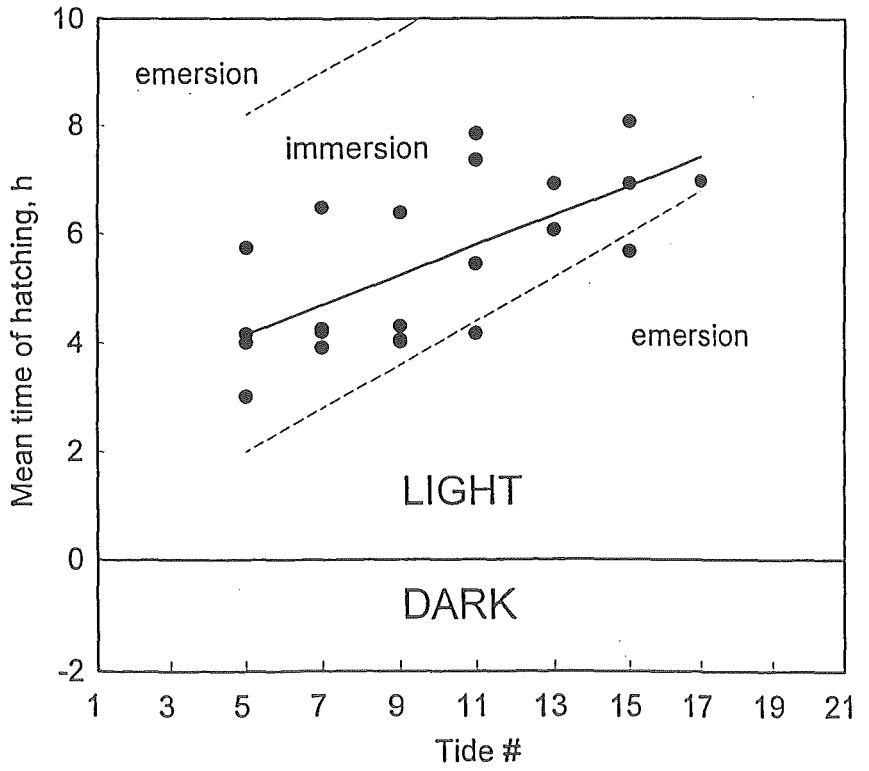


Figure 3.8 *Cyclograpsus lavauxi*. Regression of mean release times on consecutive high tides (at day time) for data in Figure 3.6. Dashed line represents the start and end of immersion periods corresponding to the previously entrained tidal cycle. Time zero represents start of dark phase. Each point) (n = 20) represents the mean hatching time of a single crab (several thousand larvae released over 3-5 h). The line represents the regression y = 0.272(x) - 2.79.

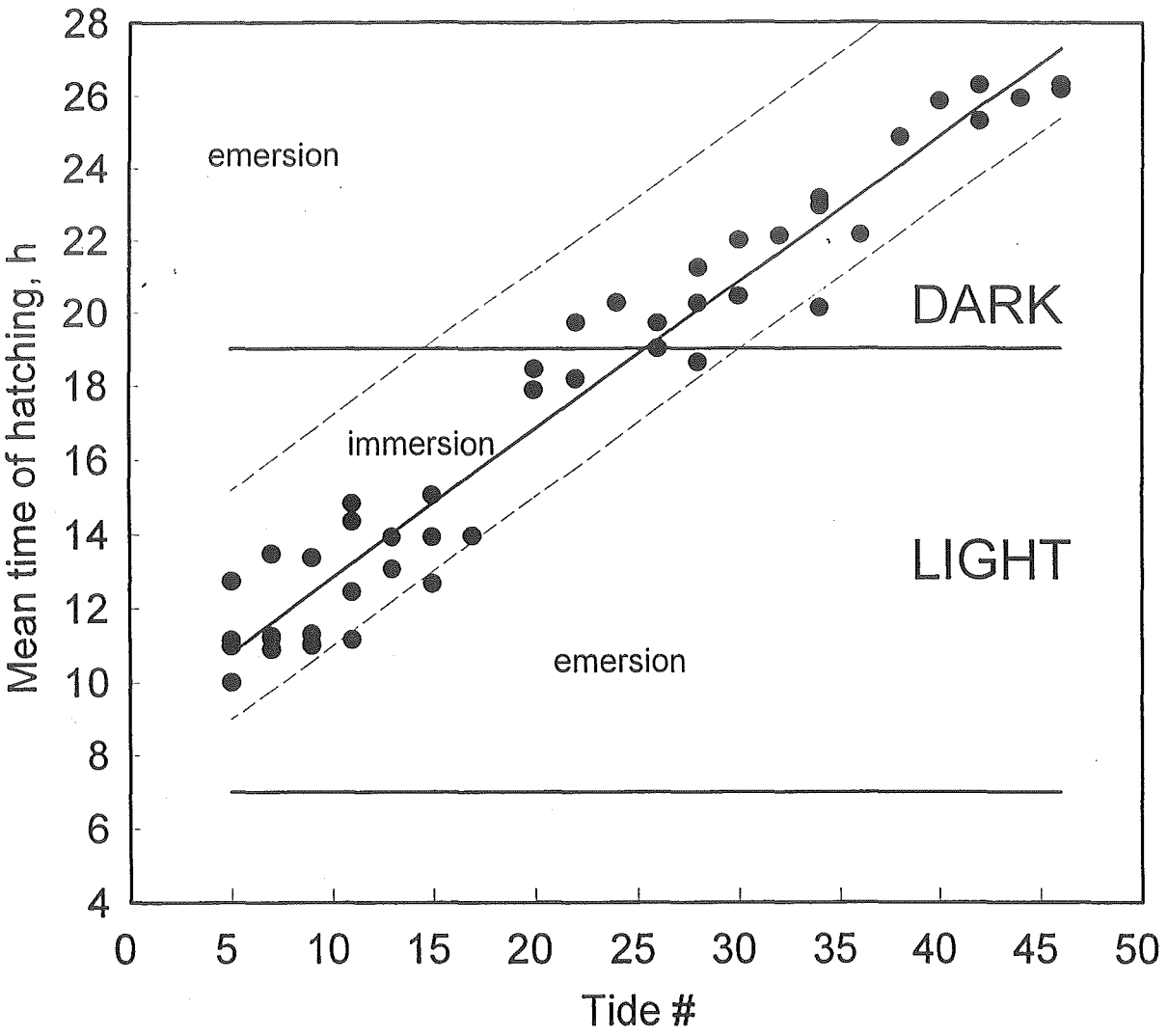


Figure 3.9 *Cyclograpsus lavauxi*. Regression of mean release times on consecutive high tides both at day and night time. Light phase and dark phase start at 7.00 a.m. and 7.00 p.m. respectively (NZDT). Data from Figure 3.5 and 3.6 are combined and plotted together. Dashed line represents the start and end of immersion periods corresponding to the previously entrained tidal cycle. Each point (n = 44) represents the mean hatching time of a single crab (several thousand larvae released over 3-5 h). The line represents the regression y = 0.402(x) - 8.7966.

Table 3.3 Comparison of mean ± SE of total number and percentage of hatching between attached and detached eggs of *Heterozius rotundifrons* under 12 hL : 12 hD cycle. Time period of observation was 4-6 days for each crab. All attached eggs were hatched as swimming larvae.

Crab No.	Attached eggs		Detached eggs			
	Number	% hatched	Number	% hatch (swimming)	% hatched (non-swimming)	% unhatched
1	196	100	131	14.50	19.08	66.41
2	214	100	165	15.76	22.42	61.82
3	110	100	85	20.00	17.65	62.35
4	224	100	168	13.69	20.24	66.07
5	183	100	166	15.06	27.71	57.23
6	144	100	72	23.61	29.17	47.22
7	157	100	104	19.23	26.92	53.85
8	134	100	119	18.49	21.01	60.50
9	208	100	168	12.50	16.07	71.43
10	217	100	180	15.56	18.33	66.11
11	155	100	128	13.28	25.78	60.94
Mean ± SE		100 ± 0		16.52 ± 1.03	22.22 ± 1.36	61.27 ± 2.02

Table 3.4 Comparison of mean ± SE of total number and percentage of hatching between attached and detached eggs of *Cyclograpsus lavauxi* under 12 hL : 12 hD cycle. All hatched were swimming larvae.

Crab No.	Attached eggs		Detached eggs			
	Number	% hatched	Number	%hatched (same time as attached)	% hatched (24 hrs)	% unhatched
1	3912	100	3484	3.62	24.57	71.81
2	5097	100	4764	3.92	25.90	70.17
3	6536	100	5940	1.90	18.15	79.95
4	2869	100	2554	4.78	29.91	65.31
5	8008	100	7009	3.65	22.00	74.35
6	4812	100	3462	3.49	25.88	70.62
7	2718	100	1612	2.73	16.62	80.64
Mean ± SE		100 ± 0		3.44 ± 0.34	23.29 ± 1.77	73.27 ± 2.08

DISCUSSION

Heterozius rotundifrons and *Cyclograpsus lavauxi* both show rhythms in larval release when they were acclimated to a new environment in laboratory. Release occurred in relation to the new LD and tidal cycles in both species. For *H. rotundifrons*, there seems to be a complex interaction of hatching with light cycle and tidal cycle. Hatching in this species is concentrated around the immersion time at night. However, it was found that hatching was advanced as the immersion time occurred later in the dark phase. The adaptive significance of this could be that the crabs maximise the period of darkness following hatching. For *C. lavauxi*, larval release is primarily related to the tides, release occurring during the period of immersion, regardless of whether it was day or night. In the experiments equal immersion and emersion periods were used. However, for this species, presumably their period of immersion is much shorter at the top of the shore and there is little scope for drift behind the tide to maximise darkness. Indeed they make equal use of daylight tides.

Female crabs of *H. rotundifrons* released larvae over 4-6 consecutive nights, whereas, in *C. lavauxi*, larval release occurred only once and when it began, all of the larvae were released within a few hours. It has been reported that for warm temperate and tropical brachyurans, egg hatching is a short duration event, with all eggs present on a female usually hatching within 15-30 min (DeCoursey, 1979; Forward *et al.*, 1982). Exceptions occur especially among the Xanthidae which occasionally release a group of larvae each night for two or three consecutive nights (Forward *et al.*, 1982; Christy, 1986; De Vries and Forward, 1989). Since *Heterozius rotundifrons* belongs to the family Belliidae which is closely related to Xanthidae (McLay, 1988). It is not surprising that its hatching characteristics are more similar to the xanthids than other brachyurans. It has been found that development of eggs of *H. rotundifrons* were not synchronized within the same egg mass (observation from the embryonic developmental study in chapter 2). This may explain why hatching in this species occurred for 4-6 consecutive nights. It is thus possible that larvae that hatched at the same night have morphological maturity synchronised. Only larvae that are ready to hatch would be released during that night and others that nearly hatch would not be released until the next period of hatching 24 hours later. However, the question of how hatching is regulated so that small batches of larvae can be hatched night after night over a period as long as 4-6 days may be explained by an endogenous component acting on the larvae. It is possible that embryos within the eggs of *H. rotundifrons* also have the same endogenous rhythm as adult crabs. They probably are active only at night time high tide as adult crabs. It is possible that

the strong movements of some body parts of embryos occurred only at night time high tide which probably caused the breakage of the egg membrane. The similar reason can be used to explain the hatching of *C. lavauxi* which occurred during high tide both at day and night time. Adults of this species are active during the period of high tide either day or night time and this rhythmic behaviour is probably also found in embryos within the eggs. Thus, it is possible that hatching in this species occurred during high tide period when embryos have strong movements and some body parts of them break the membrane. However, in this species larval release occurred as one burst only. This is because of the synchronization of development within the same egg mass (observation from the embryonic developmental study in chapter 2).

Previous studies have led to different conclusions on the timing of hatching in crustaceans as to whether it is controlled by the embryo itself, the female, or both. Pandian (1970) suggested that a clock that sets the hatching time is within the egg. Ennis (1973) also proposed an endogenous factor controlling the timing of hatching, but was inconclusive about whether it is in the embryo or in the mother. Brandford (1978) reported that in *Homarus*, eggs removed more than 10 days prior to larval release still hatched. He also stated that rhythmic release of larvae of *Homarus gammarus* at night was partly controlled by an endogenous component in the female. Hatching of attached eggs was rhythmic in natural LD, occurring just after sunset, while hatching was arrhythmic for unattached eggs. In the estuarine crab *Rhithropanopeus*, hatching occurred when the embryos were separated 1-5 days before larval release by the female (Forward and Lohmann, 1983). Similarly, in *H. rotundifrons* and *C. lavauxi*, embryos can complete their development and hatch as viable larvae even when they are separated from the mother. However, the percentages of hatching in detached eggs of both species were very low. Hatching of detached eggs of both species was found to be less synchronized than attached eggs. The reduced hatching success and unsynchronisation of hatching in detached eggs probably indicates the importance of parental assistance in the hatching process. Forward and Lohmann (1983) suggested that the timing of hatching is controlled by the embryo, and that the role of the female is to synchronize embryonic development. However, it is possible that some kind of damage associated with removal may cause the percentage of hatching in detached eggs to be decreased.

In both species, it was found that some hatched larvae did not swim or died soon after hatching and some unhatched eggs also died. Embryo mortality, caused by microbial fouling of the egg surface, has been a problem in culturing externally brooding decapods (Fisher, *et al.* 1976) and most decapods exhibit parental care in aeration and grooming of embryos, by picking and brushing the egg mass with specially adapted pereiopods

(Bauer, 1979). Coffin (1958), rearing the larvae of *Pagurus samuelis* (Stimpson), noted that unless the water was changed frequently immediately prior to hatching the zoeae would emerge in a weakened condition or die soon after liberation. In standing water, even though aerated, the possibility of increasing the growth of deleterious fungi, such as *Lagenidium callinectes* Couch, is enchanced. Previous workers (Sandoz and Rogers, 1944) have noted that fungus infected eggs either failed to hatch or produced "pre-zoea" which died after 48 hours. Thus, it possible that embryo and egg mortalities of *H. rotundifrons* and *C. lavauxi* resulted from some microbes or fungi which fouled the egg surface perhaps affecting O_2 diffusion into the egg.

In conclusion, there was a precise timing of larval release for *H. rotundifrons* and *C lavauxi.* In *H. rotundifrons*, the timing of larval release occurred in association with both the LD and tidal cycles, whereas it was correlated only with the tidal cycle in *C. lavauxi.* Hatching in *H. rotundifrons* occurred for about 4-6 consecutive nights, whereas it occurred only once within a few hours in *C. lavauxi.* Detached eggs of both species were able to hatch without the parental care of females but hatching was less synchronized in detached eggs than in the attached eggs in both species. Percentages of hatched larvae from detached eggs in both species were very low. For *H. rotundifrons*, hatching occurred for several nights would give more opportunitiy for the larvae to survive more than those of *C. lavauxi* which occurred within one night. Larval hatching during high tide, particularly at night, would be a behavioral mechanism for reducing predation on the adults. Hatching rhythms during the high tide period may be adaptive not only for adult crabs but for dispersion of larvae into the sea (Christy, 1978; Saigusa and Hidaka, 1978; Wheeler, 1978). Planktonic larval forms may be carried away from suitable substrates by currents (Wood and Hargis, 1971; de Wolf, 1973). Releasing at a particular time presumably allows enhanced reproductive efficiency through increased chances of survival of larvae.

CHAPTER FOUR

OXYGEN CONSUMPTION OF OVIGEROUS CRABS AND DEVELOPING EGGS

INTRODUCTION

Many studies have been made on the physiology of gas exchange in decapod crustacean (Johansen *et al.*, 1970; Newell *et al.*, 1972; Taylor and Butler, 1978; Hawkins *et al.*, 1982; Taylor and Davies, 1982; Houlihan and Innes, 1984; O'Mahoney and Full ,1984; Innes *et al.*, 1986; Eshky *et al.*, 1988; Al-Wassia *et al.*, 1989; Erdman *et al.*, 1991). Among these studies, bimodel gas exchange has been shown to occur in a variety of intertidal crabs (Newell *et al.*, 1972; Taylor and Butler, 1978; Hawkins *et al.*, 1982; Houlihan and Innes, 1984; Innes *et al.*, 1986). The capacity for oxygen uptake in both air and water of these animals is however variable and profoundly influenced by environmental and endogenous factors (Newell, 1973). The endogenous factors which influence respiration rate are body size, activity level, sex, age, nutrition and reproduction (Newell, 1973 and Dejours,1981).

Unlike most vertebrate animals, some invertebrates, especially crustaceans, carry their broods by attaching them outside their bodies. By separating the respiration rates of embryos and brooding females of *Daphnia*, Glazier (1991) found no detectable energetic cost of carrying a brood. In crabs, ovigerous females are found carrying developing eggs on the ventral surface attached to pleopods. Although there have been numerous studies on the physiology of respiration of crabs, until recently, there have been no reports concerning the relationships between the respiration rates of the embryos and brooding females. However, Wheatly (1981) investigated the relationship between ventilatory behaviour of ovigerous female shore crabs (*Carcinus maenus*) and the oxygen consumption of the developing eggs in relation to lowered ambient oxygen tension. She found that under hypoxic conditions, ovigerous females exhibited an "emersion" response in which the usual direction of ventilation is reversed. This emersion behaviour differs from the typical emersion behaviour found in non-ovigerous crabs. Streams of air bubbles did not emanate from the Milne-Edwards openings, instead, it is directed from the openings at the base of posteriormost pair of walking legs and thus over the developing egg mass. Moreover, she found that ovigerous females exhibited emersion behaviour at P_IO_2 that was significantly higher than non-ovigerous crabs. This behaviour has been modified to confer a possible respiratory advantage not only to the parent animal but also on the eggs. Other possible mechanisms of ventilating the eggs

involve movements of the abdomen or pleopods but there is little information on the physiological advantage of these adaptations. During brooding, the oxygen requirements of ovigerous crabs might increase due to either the work of carrying the eggs, or the increase in activity associated with supplying oxygen to the eggs, or the metabolic rate of the developing eggs.

Heterozius rotundifrons and *Cyclograpsus lavauxi* occupy different positions in the intertidal zone (low-shore and high-shore respectively). Ovigerous females of these species are of similar size (about 2 g) and carry similar masses of eggs (typically 0.2-0.3 g) on the abdominal pleopods. However, in *H. rotundifrons* the brood consists of only a few hundred large yolky eggs (egg volume increasing from about 240 to 360 nL at hatching) which take 6 months to develop and hatch in the laboratory at 15°C. In contrast, *C. lavauxi* carry several thousands of eggs (volume 9 to 19 nL) which hatch in only 2 months.

Since *H. rotundifron*s and *C. lavauxi* occupy different positions on the shore and hence differ in exposure to air and water in the tidal cycle, the first objective of this study was to provide information on the overall oxygen requirements in air and water of ovigerous females of these two species of intertidal crabs. Oxygen consumption by the eggs at different developmental stages was also measured in order to assess the component of total respiration attributable to the eggs. Since *H. rotundifrons* (few large eggs) and *C. lavauxi* (many small eggs) adopt different strategies with respect to their energetic investment in embryonic development, it was of interest to compute the total cost of development of a single egg in terms of oxygen uptake. This study also presented an opportunity to compare the metabolic rates both of individuals of different developmental stages, within and between species, for body masses differing by many orders of magnitude. It will be shown that for the developing eggs of these crabs, the usual allometric relationship does not apply. Additionally, measurements of oxygen consumption at timed intervals during development from spawning to hatching were combined to estimate the total cost of development in terms of oxygen uptake for these two species of differing egg size and development time. Responses of the eggs and ovigerous crabs to hypoxia will be reported in the following chapter.

MATERIALS AND METHODS

Animals

Non-ovigerous and ovigerous *H. rotundifrons* and *C. lavauxi* were collected from the intertidal zone at Kaikoura (see chapter 2 for details of location). Each species of crab was collected twice for these experiments. Non-ovigerous and ovigerous crabs with early stage (blastula) eggs were collected during the spawning period in April for *H. rotundifrons* and during mid November for *C. lavauxi.* Ovigerous crabs with late stage (two lobes of yolk) eggs of both species were collected simultaneously in January. *H. rotundifrons* were found half buried in sand and gravel under stones and pebbles in lower shore, whereas *C. lavauxi* were collected from under various boulders around high tide level. The crabs were transported to and maintained in an aquarium at the Department of Zoology, University of Canterbury. Crabs were acclimated for one week prior to experimentation in polypropylene tanks with recirculating seawater, mean salinity 35 ppt with a mean air temperature 15°C under a 12:12 LD cycles. During this period, crabs were starved and given access either air or seawater.

Aerial oxygen consumption

Aerial rates of oxygen consumption of individual crabs were measured using a constant pressure differential respirometer as described by Davies (1966). The whole apparatus consisted of three main parts: 1) Perspex respiration and compensation chambers 2) the manometer block and 3) the micrometer. The operating system of this respirometer was based upon the constant-pressure principle in which initially the pressure was the same in both chambers. The CO_2 produced was absorbed by KOH separated from the animal by a small plastic bottle through which enough holes were drilled to allow free circulation of the air. When oxygen was consumed by the respiring animal in the respiration chamber a decrease in pressure resulted. The pressure difference between the respiration chamber was indicated by the upward movement of fluid in the respiration chamber side of manometer. The micrometer was then screwed down, advancing the spindle into the respirometer chamber until the two arms of the manometer were again level. At this stage the pressure was the same in both chambers and the volume advanced by the spindle was therefore equivalent to the volume of oxygen consumed.

The rates of oxygen consumption of non-ovigerous, early stage ovigerous and late stage ovigerous crabs were measured at rest. Each crab was blotted dry and put into a respirometer immersed in a water bath at the experimental temperature (15°C). The system was allowed equilibrate temperatures Because disturbance caused by handling the crab when placing it into the respirometer resulted in elevated rates of oxygen consumption, crabs were allowed to settle in the apparatus for 3-5 hours. When measurements were to be taken, the respirometer was closed off. Readings were taken at 20 min intervals and the rate of oxygen consumption was calculated over three hours period as crab was in a steady resting state. After the measurements were completed, the crab was removed from respirometer and weighed. After weighing, the eggs were removed and weighed and the number of eggs was determined (in some cases by counting a weighed subsample).

Rates of oxygen consumption in air of individual crab was calculated from the slope of regression line plotted between micrometer reading and time, multiplied by calibration factor of aerial respirometers. The value was then corrected to standard conditions of 0°C and 760 mmHg (S.T.P.) and converted to weight-specific, molar hourly oxygen consumption ($\dot{M}o_2$) as follow :

$$\dot{M}o_2 = \frac{S \times C \times (273/T) \times (B/760) \times (60/W)}{22.4} \qquad \mu mol.g^{-1}.h^{-1}$$

where, S is the slope of regression line plotted between micrometer reading and time ($mm.min^{-1}$); C is the calibration factor of aerial respirometer (1 mm = 31.67 µL); T is the waterbath temperature (°K); B is the atmospheric pressure (torr) and W is the mass of crab (g).

Aquatic oxygen consumption

Aquatic rates of oxygen consumption of individual crabs at rest were carried out using a closed-system respirometer. The whole system consisted of four respirometer chambers (one respirometer was used for a blank), a peristaltic pump and a 5 L aerated sea water reservoir. Four plastic bottles with a volume of 250 mL were used as respirometer chambers. The sea water within the chambers was continuously circulated using a peristaltic pump and three-way taps, which allowed aerated sea water from the reservoir to flow into the chambers. The water was returned to the reservoir via an overflow tube. When measurement was started, the taps were switched to recirculate

water through the chambers without disturbing the animals. The rates of oxygen consumption were measured in terms of the change in Po_2 of the sea water using a Radiometer oxygen electrode. The oxygen electrode was calibrated using a solution of 0.01 molar sodium borate and sodium sulphite to provide a solution having a Po_2 of zero, and against water-saturated air at the experimental temperature.

Measurements were made in non-ovigerous, early-ovigerous and late-ovigerous crabs. The four respirometers were filled to overflowing with pasteurised, air-saturated sea water and a crab was placed in each of three respirometers and another respirometer was left as a blank. With the pump turned on and the three-way taps opened, bungs were inserted so that no air was trapped in each chamber and water overflowed out into the reservoir. The taps were closed off to allow water to flow through to completely purge the system of air, then the taps were opened. Circulation within the respirometers were maintained using a peristaltic pump connected to the respirometers. The respirometers were immersed in a water bath at 15°C and clamped securely in position. Three to five hours was allowed for temperature equilibration and for the crabs to settle before measurements. When measurements were to be taken, the taps were closed off and a 1 mL water sample was taken from the respirometers. Po_2 in the water samples were measured using a thermostatted oxygen electrode (Radiometer) and blood gas/acid/base monitor (Radiometer PHD 71). Po_2 was then measured again and the rates of oxygen consumption were calculated at three hour periods as crabs were in a steady resting state. After the measurements were completed, crabs were removed from respirometers and weighed. The eggs were then removed and weighed and the number of eggs determined (in some cases by counting a weighed subsample).

To obtain the weight specific oxygen consumption in water of individual crabs, the difference in ΔPo_2 between the sample and the blank were corrected using the oxygen solubility coefficients at water bath temperature and by converting the amount of oxygen consumed as follows :

$$\dot{M}o_2 = \frac{(\Delta Po_2 sample - \Delta Po_2 blank) \times a \times V \times 60}{t \times W} \qquad \mu mol.g^{-1}.h^{-1}$$

where, a is the solubility of oxygen in seawater at waterbath temperature (= 1.6218 $\mu mol.L^{-1}$ $torr^{-1}$ at temperature 15°C); V is the volume of aquatic respirometer (L); t is the experimental time (min) and W is the mass of crab (g).

Oxygen consumption of developing eggs.

The oxygen uptake of developing eggs was measured using a respiration cell of volume 1 mL. The cell was formed from a precision blown glass tube fused into a glass water jacket thermostatted at 15°C. A microcathode electrode (Strathkelvin Instruments 781b) was fitted into the side of the cell and eggs were introduced into the respiration cell through a small hole on the top of the chamber closed by a small glass stopper. A magnetic spinbar within the respiration cell was controlled by a magnetic stirrer. Oxygen consumption by the eggs produced a decrease in Po_2 which was displayed on an oxygen meter (Strathkelvin Instruments 781b). The oxygen uptake of the eggs was calculated from the Po_2 change.

The rates of oxygen consumption of developing eggs were measured from groups of eggs (50 eggs for *H. rotundifrons* and about 1,000 eggs for *C. lavauxi*) separated from the egg masses of the crab. Eggs were carefully removed from the setae on the endopodites of the abdominal pleopods and transferred to the respiration cell. The cell was then closed off and the eggs were allowed to equilibrate for 5 min before taking the first Po_2 measurement. Measurements were taken again after 24 min. The quantity of oxygen consumed by developing eggs was obtained from the difference between ΔPo_2 of eggs and a blank run of similar duration. The weight specific oxygen consumption of developing eggs, the oxygen uptake by individual eggs and the total cost of their development was then calculated

Rates of oxygen consumption of crab eggs in water were calculated in the same way as those in adult crabs. To obtain the weight specific oxygen consumption ($\dot{M}o_2$), the difference in ΔPo_2 between the crab eggs and the blank was corrected using the oxygen solubility coefficients at water bath temperature and by converting the amount of oxygen consumed as follows :

$$\dot{M}o_2 = \frac{(\Delta Po_2 eggs - \Delta Po_2 blank) \times a \times V \times 60}{t \times W} \qquad \mu mol.g^{-1}.h^{-1}$$

where, a is the solubility of oxygen in seawater at waterbath temperature (= 1.6218 μmol L^{-1} $torr^{-1}$ at temperature 15°C); V is the volume of respiration cell (L); t is the experiment time (min) and W is the whole mass of crab eggs (g).

Data analysis

Mass-specific metabolic rates of animals typically decrease with increasing body mass according to the allometric relation

$$\dot{M}o_2 = aW^b \qquad \mu mol.g^{-1}.h^{-1}$$

where a is the metabolic rate for an animal of unit mass (1 g in this case), W is the mass (g) and b is the mass exponent (usually negative).

As shown below, this negative dependency of metabolic rate on mass holds for non-ovigerous and ovigerous crabs of both species. Although the mass range of ovigerous crabs collected in this study was quite narrow (about 1.5 g - 3.0 g) it was desirable to remove variability from this source and to allow for this effect in comparison among groups of crabs (between species, non-ovigerous versus ovigerous of differing egg stages) of differing mean mass.

Thus, mass-specific metabolic rates are illustrated below by plotting against body mass and the values of a and b in the above equation were obtained by double logarithmic regression. Differences in mean $\dot{M}o_2$ between treatment groups were tested by analysis of covariance and including mass as a covariate. Both variables were transferred to logarithms. Pairwise comparisons among treatment were made using Scheffe (post hoc) test.

Differences in mean metabolic rate among groups of crabs would be indicated by differences in the value of a, the estimated metabolic rate for a 1 g crab. As, this mass falls outside the range for ovigerous crabs used in this study, a more meaningful statistical comparison was obtained from estimates of the mean metabolic rate of 2 g crabs which was close to the mean mass of all treatment groups. Thus, individual mass-specific metabolic rates were scaled to 2 g mass using the individual mass exponents (b) for each treatment group determined as above (in Table 4.1)

$$\dot{M}o_{2(2)} = \dot{M}o_{2(w)} \times \frac{2^b}{W^b} \qquad \mu mol.g^{-1}.h^{-1}$$

where $\dot{M}o_{2(2)}$ is the scaled value, $\dot{M}o_{2(W)}$ is the measured value and W is the mass of the

crab (g). Populations of scaled values were then compared using one-way analysis of variance or students t-test. A probability of 0.05 was taken to indicate statistical significance unless specifically indicated otherwise.

RESULTS

Aerial and aquatic oxygen consumption of non-ovigerous, early-ovigerous and late-ovigerous Heterozius rotundifrons and Cyclograpsus lavauxi.

$\dot{M}o_2$ decreased with the mass of both species of non-ovigerous and ovigerous crabs used in the experiments. Mass specific oxygen consumption in air of non-ovigerous *H. rotundifrons* was similar to that in seawater and these rates were less than the rates in air found in non-ovigerous *C. lavauxi*. Non-ovigerous *C. lavauxi* showed higher (almost twice) mass specific oxygen consumption in air than in seawater. (Figure 4.1 and Table 4.1). When comparing values on an analysis of covariance and a Scheffe's statistical test, there proved to be a significant difference between mean $\dot{M}o_2$ in both air and seawater of *H. rotundifrons* and those in air of *C. lavauxi* (Table 4.2).

Mass specific rates of oxygen consumption in both air and seawater of late-ovigerous *H. rotundifrons* and *C. lavauxi* were higher than in non-ovigerous crabs (Figure 4.2-4.5 and Table 4.1). Analysis of covariance and a Scheffe's statistical test showed that mean $\dot{M}o_2$ both in air and seawater of late-ovigerous *H. rotundifrons* and *C. lavauxi* were significant higher than in the corresponding non-ovigerous crabs (Table 4.3).

Similar results were found when individual mass specific metabolic rates of crabs were scaled to 2g mass. Mass specific oxygen consumption in both air and seawater of late-ovigerous *H. rotundifrons* and *C. lavauxi* were higher than in non-ovigerous crabs (Figure 4.6). The statistical significance of mass specific oxygen consumption of scaled values were compared using one way analysis of variance and Scheffe's pairwise comparison. It was found that $\dot{M}o_2$ in both air and seawater of late-ovigerous *H. rotundifrons* and *C. lavauxi* were significant higher than those of non-ovigerous crabs (Figure 4.6).

Oxygen consumption of developing eggs of Heterozius rotundifrons

Table 4.4 and Figure 4.8 shows mean values (± S.E.) of mass specific oxygen consumption in seawater of eggs of *H. rotundifrons* at different stages of their development measured over 24 min after a short period of acclimation in the respirometer at 15°C. Mass specific oxygen consumption of eggs increased with their

Table 4.1 Regression equations for weight specific oxygen consumption ($\mu mol.g^{-1}.h^{-1}$) in air and seawater of non-ovigerous and ovigerous *Heterozius rotundifrons* and *Cyclograpsus lavauxi* after 3 hours settling at 15°C.

Respiration	Species	Status	Regression equation	r	n
Aerial	*Heterozius rotundifrons*	Non-ovigerous	$\dot{M}o_2 = 0.90\ W^{-0.520}$	-0.653537	15
		Early-ovigerous	$\dot{M}o_2 = 0.92\ W^{-0.168}$	-0.317100	15
		Late-ovigerous	$\dot{M}o_2 = 1.41\ W^{0.010}$	-0.012640	8
	Cyclograpsus lavauxi	Non-ovigerous	$\dot{M}o_2 = 1.89\ W^{-0.113}$	-0.416731	11
		Early-ovigerous	$\dot{M}o_2 = 2.15\ W^{-0.054}$	-0.189199	5
		Late-ovigerous	$\dot{M}o_2 = 3.16\ W^{-0.173}$	-0.608164	11
Aquatic	*Heterozius rotundifrons*	Non-ovigerous	$\dot{M}o_2 = 0.84\ W^{-0.327}$	-0.217166	18
		Early-ovigerous	$\dot{M}o_2 = 1.19\ W^{-0.446}$	-0.477840	18
		Late-ovigerous	$\dot{M}o_2 = 2.37\ W^{-0.511}$	-0.694356	7
	Cyclograpsus lavauxi	Non-ovigerous	$\dot{M}o_2 = 1.02\ W^{-0.162}$	-0.007582	15
		Early-ovigerous	$\dot{M}o_2 = 1.09\ W^{-0.043}$	-0.056994	8
		Late-ovigerous	$\dot{M}o_2 = 1.71\ W^{-0.003}$	-0.459396	9

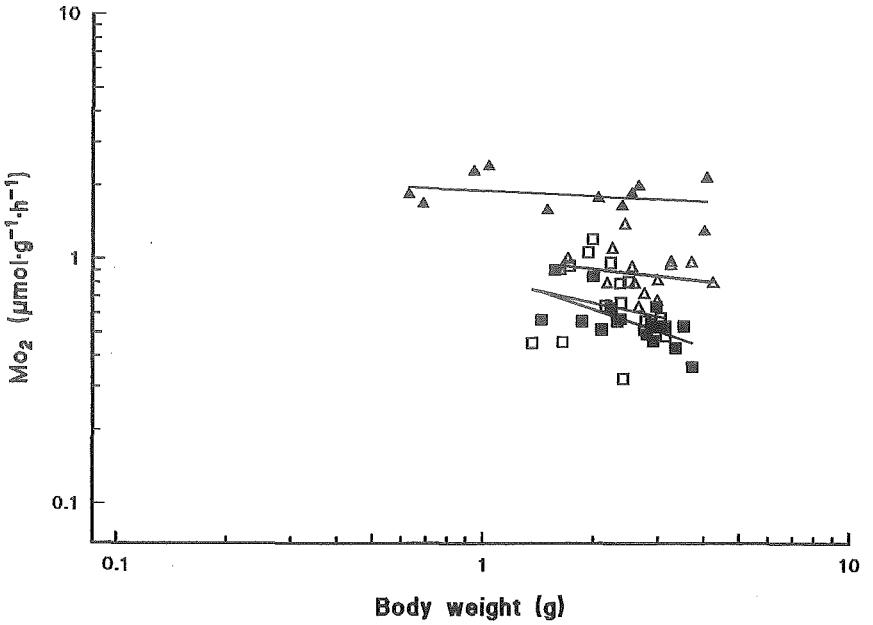


Figure 4.1 The relationship between mass specific oxygen consumption and body mass of inactive non-ovigerous, *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, in air and seawater after 3 hours settling at 15°C. ■, *H. rotundifrons* in air ($\dot{M}o_2 = 0.90\ W^{-0.521}$); □, *H. rotundifrons* in seawater ($\dot{M}o_2 = 0.84\ W^{-0.327}$); ▲, *C. lavauxi* in air ($\dot{M}o_2 = 1.89\ W^{-0.113}$) and △, *C. lavauxi* in seawater ($\dot{M}o_2 = 1.02\ W^{-0.162}$).

Table 4.2 Analysis of covariance and Scheffe's statistical test of significance in mean $\dot{M}o_2$ between non-ovigerous *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, in air and seawater, at 15°C.

Comparison	*P*
Aerial (*H. rotundifrons)* and Aquatic (*H. rotundifrons)*	> 0.600
Aerial (*C. lavauxi*) and Aquatic (*C. lavauxi*)	< 0.001*
Aerial (*H. rotundifrons)* and Aerial (*C. lavauxi*)	< 0.001*
Aquatic (*H. rotundifrons)* and Aquatic (*C. lavauxi*)	> 0.400

* Asterisks indicated the significant differences in mean $\dot{M}o_2$ between two comparing groups

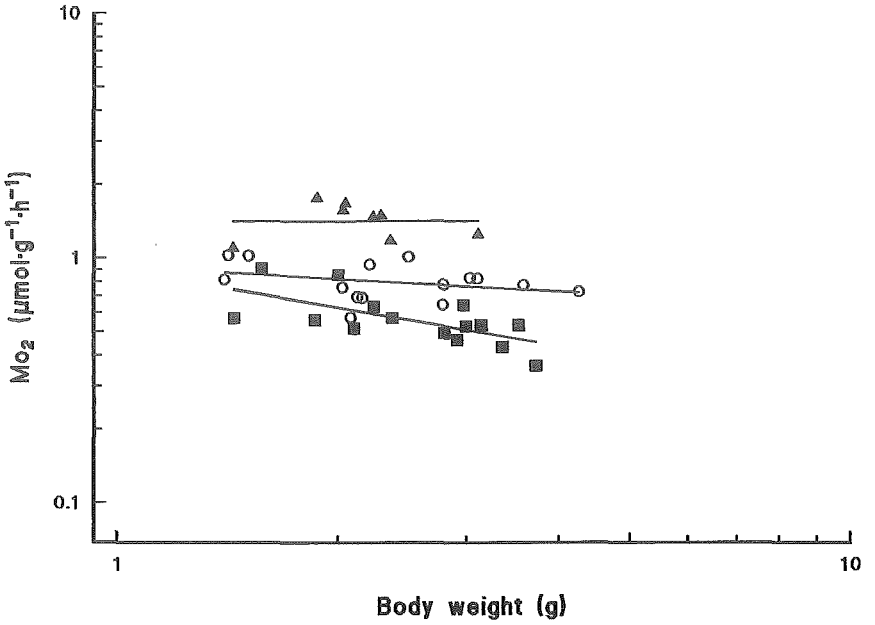


Figure 4.2 The relationship between mass specific oxygen consumption in air and body mass of inactive non-ovigerous, ■ ($\dot{M}o_2 = 0.90\ W^{-0.521}$); early-ovigerous, o ($\dot{M}o_2 = 0.92\ W^{-0.168}$) and late-ovigerous, ▲ ($\dot{M}o_2 = 1.41\ W^{0.010}$) of *Heterozius rotundifrons*, after 3 hours settling at 15 °C.

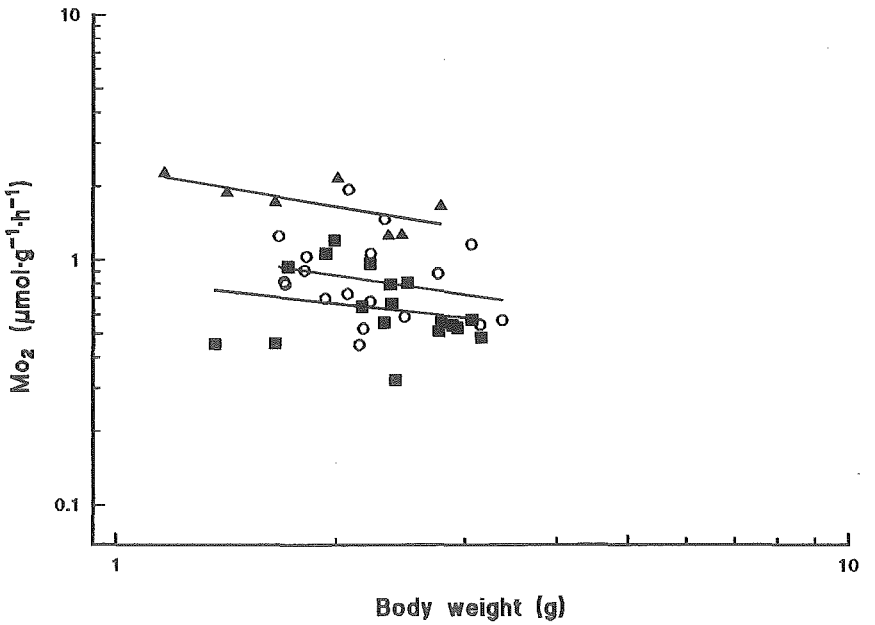


Figure 4.3 The relationship between mass specific oxygen consumption in seawater and body mass of inactive non-ovigerous, ■ ($\dot{M}o_2 = 0.84\ W^{-0.327}$); early-ovigerous, o ($\dot{M}o_2 = 1.19\ W^{-0.446}$) and late-ovigerous, ▲ ($\dot{M}o_2 = 2.37\ W^{-0.511}$) of *Heterozius rotundifrons*, after 3 hours settling at 15 °C.

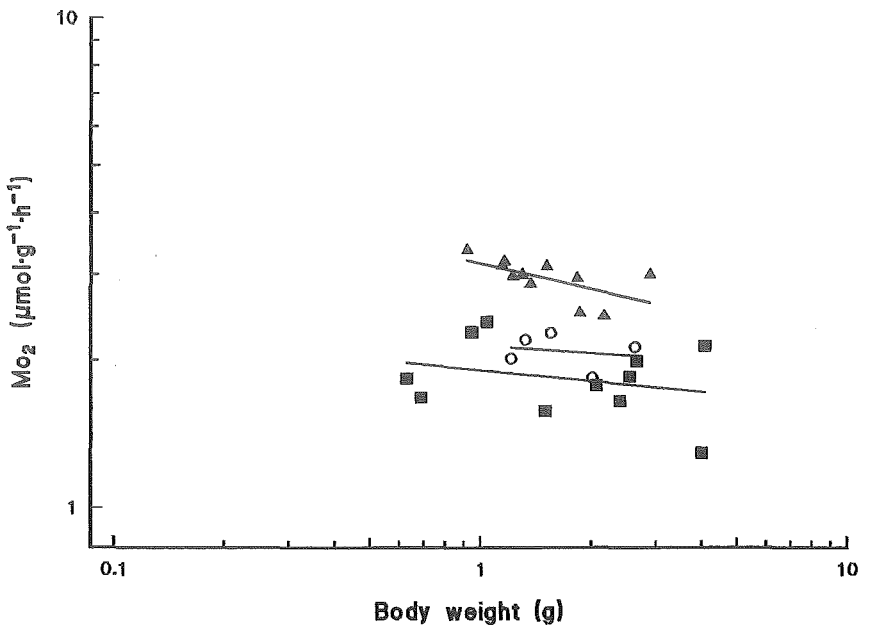


Figure 4.4 The relationship between mass specific oxygen consumption in air and body mass of inactive non-ovigerous, ■ ($\dot{M}o_2 = 1.89\ W^{-0.113}$); early-ovigerous, o ($\dot{M}o_2 = 2.15\ W^{-0.054}$) and late-ovigerous, ▲ ($\dot{M}o_2 = 3.16\ W^{-0.173}$) of *Cyclograpsus lavauxi*, after 3 hours settling at 15 °C.

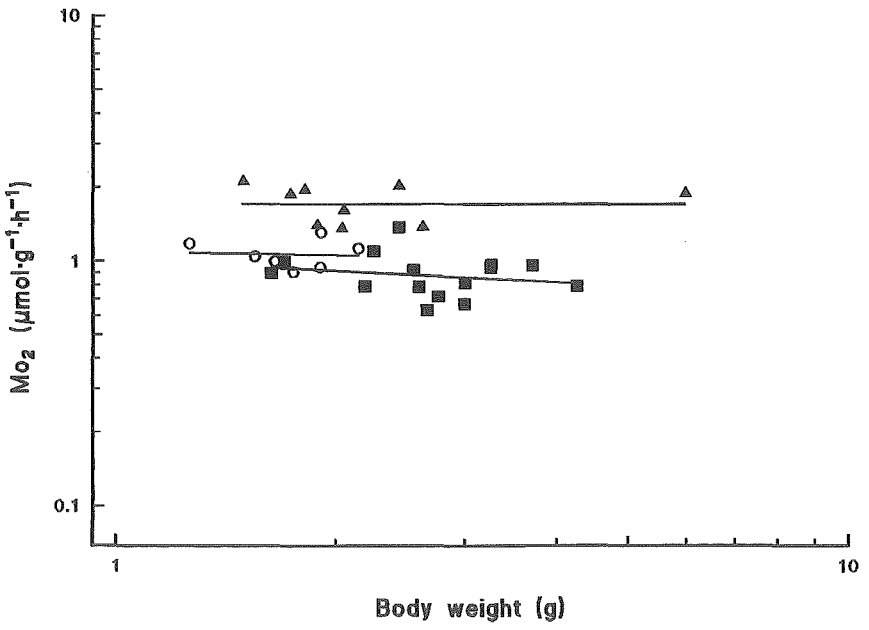


Figure 4.5 The relationship between mass specific oxygen consumption in seawater and body mass of inactive non-ovigerous, ■ ($\dot{M}o_2 = 1.02\ W^{-0.162}$); early-ovigerous, o ($\dot{M}o_2 = 1.09\ W^{-0.043}$) and late-ovigerous, ▲ ($\dot{M}o_2 = 1.71\ W^{-0.003}$) of *Cyclograpsus lavauxi*, after 3 hours settling at 15 °C.

Table 4.3 Analysis of covariance and Scheffe's statistical test of significance in mean $\dot{M}o_2$ between non-ovigerous, early-ovigerous and late-ovigerous of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*. in air and seawater, at 15°C.

Respiration	Species	Comparison	*P*
Aerial	*Heterozius rotundifrons*	Non-ovigerous and Early-ovigerous	> 0.400
		Early-ovigerous and Late-ovigerous	> 0.300
		Non-ovigerous and Late-ovigerous	< 0.010*
	Cyclograpsus lavauxi	Non-ovigerous and Early-ovigerous	> 0.900
		Early-ovigerous and Late-ovigerous	> 0.800
		Non-ovigerous and Late-ovigerous	< 0.05*
Aquatic	*Heterozius rotundifrons*	Non-ovigerous and Early-ovigerous	> 0.200
		Early-ovigerous and Late-ovigerous	< 0.010*
		Non-ovigerous and Late-ovigerous	< 0.001*
	Cyclograpsus lavauxi	Non-ovigerous and Early-ovigerous	> 0.700
		Early-ovigerous and Late-ovigerous	> 0.600
		Non-ovigerous and Late-ovigerous	< 0.001*

* Asterisks indicated the significant differences in mean $\dot{M}o_2$ between two comparing groups at $P \leq 0.05$ (*).

development. Eggs at the blastula stage had the lowest respiration rate, which is 0.72 ± 0.05 $\mu mol.g^{-1}h^{-1}$, compared with the rate at the end of their development, which is 9.12 ± 0.19 $\mu mol.g^{-1}h^{-1}$. That is $\dot{M}o_2$ increased more than 12 times while mass increased about 1.5 times. It is apparent that the rate increase is greater at the end of development than at the beginning (Figure 4.8). When comparing the difference between mean of mass specific oxygen consumption at each stages of development using one way analysis of variance and Scheffe's pairwise comparison of mean, there proved to be a significant different at 5% level between each stage of development, except for the first three stages.

The absolute values of oxygen consumption for single eggs ($\mu mol.h^{-1}$) at each stage of development is shown in Table 4.4 and Figure 4.10. On this basis, the increase in oxygen consumption is much greater (20 times). The area under the curve in Figure 4.10 represents the total cost of development of a single egg which is 1.517 $\mu mol.O_2$.

Oxygen consumption of developing eggs of Cyclograpsus lavauxi

As in *H rotundifrons*, mass specific oxygen consumption of developing eggs of *C. lavauxi* increased with their development (Figure 4.9 and Table 4.5). Eggs at the blastula stage had the lowest respiration rate, which is 1.13 ± 0.09 $\mu mol.g^{-1}.h^{-1}$, compared with the rate at the end of their development, which is 10.81 ± 0.63 $\mu mol.g^{-1}.h^{-1}$. Differences between mean values of mass specific oxygen consumption at each stage of development was tested using one way analysis of variance and Scheffe's pairwise comparison of mean. It was found that mass specific oxygen consumption of all stages of development were significantly different at a confidence level of 95%.

Oxygen consumption of a single egg per unit hour of each stage of development was calculated as a mean value ± S.E. The single egg at the early stage (blastula) and late stage (yolk 2 lobes) of this species consumed oxygen about 16 and 22 times respectively less than those of *H. rotundifrons.* The relationship between oxygen consumption of one egg and development time is shown on Figure 4.11. The area under the curve represents the total cost of development of a single egg which is 0.077 μmol of O_2.

On a mass specific basis, $\dot{M}o_2$ increased about 10 times while mass approximately doubled. Absolute $\dot{M}o_2$ for individual eggs increased about 14 times. Note that despite a 20-fold difference in egg mass between the two species, the mass-specific rates of

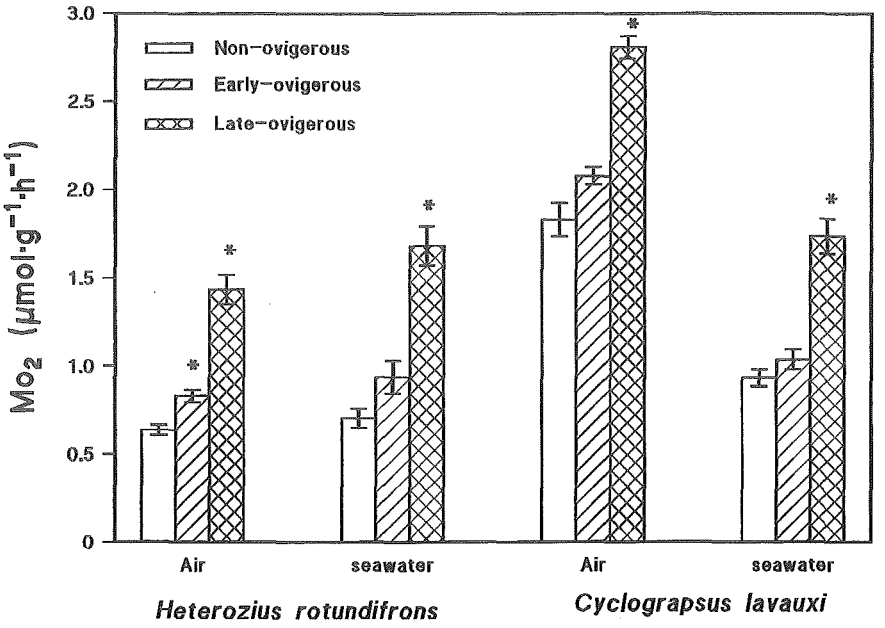


Figure 4.6 Mean (± SE) values of mass specific oxygen consumption of 2 g crabs in air and seawater of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, comparing between non-ovigerous, early-ovigerous and late-ovigerous crabs, after 3 hours settling at 15 °C. (* significantly different ($P < 0.05$) from corresponding non-ovigerous crabs).

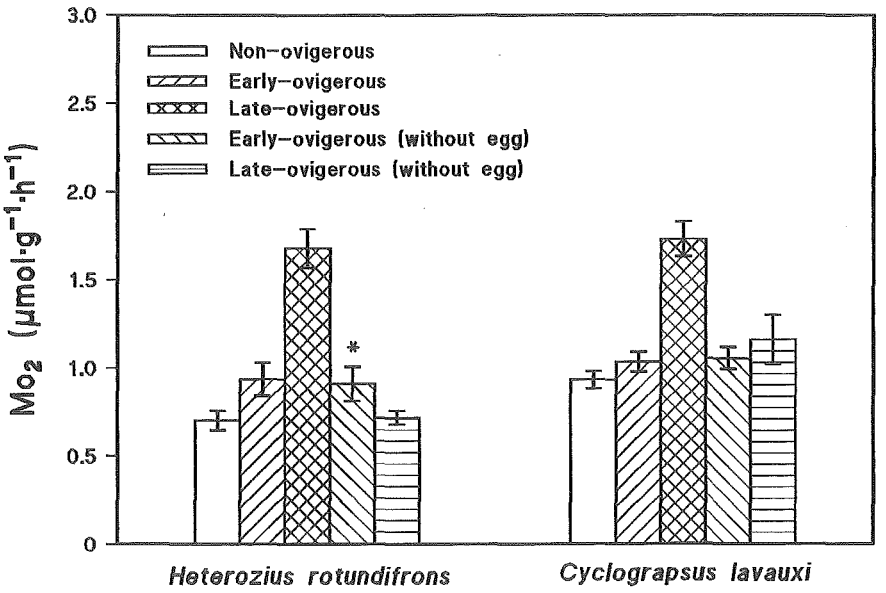


Figure 4.7 Mean (± SE) values of mass specific oxygen consumption of 2 g crabs in seawater of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, comparing between non-ovigerous, early-ovigerous, early-ovigerous (without egg), late-ovigerous and late-ovigerous (without egg) crabs, after 3 hours settling at 15 °C. (* significantly different ($P < 0.05$) from corresponding non-ovigerous crabs, other comparisons are shown in Figure 4.6).

Table 4.4 Weight specific oxygen consumption of developing eggs and oxygen consumption of single eggs of *Heterozius rotundifrons* at different stages of their development, in sea water at 15°C

Stage of eggs	Weight of one egg (mg)	$\dot{M}o_2$ ($\mu mol.g^{-1}h^{-1}$)	$\dot{M}o_2$ ($\mu mol /egg.h^{-1}$)	n
Full yolk, Late blastula (60 days)	0.269±0.003	0.72±0.05	0.000195 ± 0.000012	10
Yolk 5/6 (110 days)	0.286±0.004	1.00±0.10	0.000295 ± 0.004567	10
Yolk 4/5 (130 days)	0.315±0.010	1.45±0.06	0.000461 ± 0.000031	10
Yolk 3/5 (145 days)	0.328±0.004	2.14±0.07	0.000701 ± 0.000020	10
Yolk 1/2 (160 days)	0.362±0.002	2.98±0.08	0.001082 ± 0.000035	10
Yolk 1/4 (175 days)	0.409±0.003	5.31±0.13	0.002170 ± 0.000046	10
Yolk 2 lobes (190 days)	0.435±0.009	9.12±0.19	0.003946 ± 0.000093	10

Table 4.5 Weight specific oxygen consumption of developing eggs and oxygen consumption of single eggs of *Cyclograpsus lavauxi* at different stages of their development, in sea water at 15°C

Stage of eggs	Weight of one egg (mg)	$\dot{M}o_2$ ($\mu mol.g^{-1}h^{-1}$)	$\dot{M}o_2$ ($\mu mol / egg.h^{-1}$)	n
Full yolk, Late blastula (10 days)	0.0109±0.0008	1.13±0.09	0.0000124 ± 0.0000015	10
Yolk 5/6 (20 days)	0.0120±0.0004	2.90±0.21	0.0000155 ± 0.0000012	11
Yolk 1/2 (30 days)	0.0144±0.0004	4.48±0.15	0.0000321 ± 0.0000011	10
Yolk 1/4 (40 days)	0.0168±0.0008	6.49±0.016	0.0001087 ± 0.0000057	9
Yolk 2 lobes (55 days)	0.0203±0.0010	10.81±0.63	0.0001774 ± 0.0000194	10

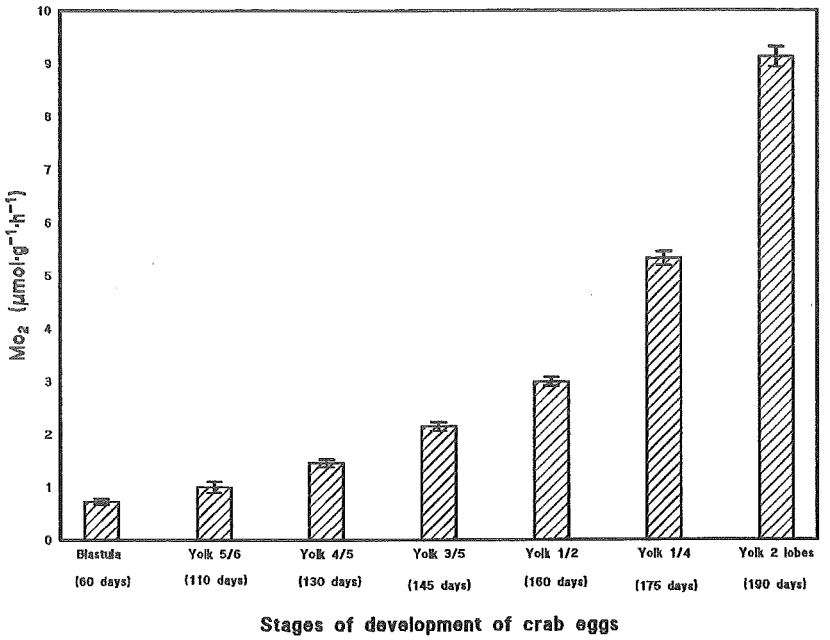


Figure 4.8 Mean values (± SE) of mass specific oxygen consumption of eggs of *Heterozius rotundifrons* at different stages of their development. The values obtained at temperature 15°C.

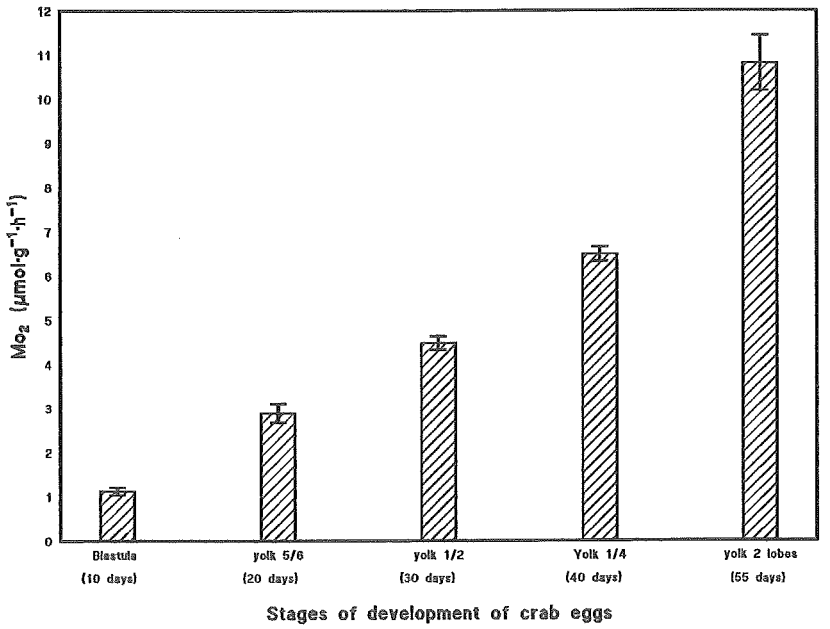


Figure 4.9 Mean values (± SE) of mass specific oxygen consumption of eggs of *Cyclograpsus lavauxi* at different stages of their development. The values obtained at temperature 15°C.

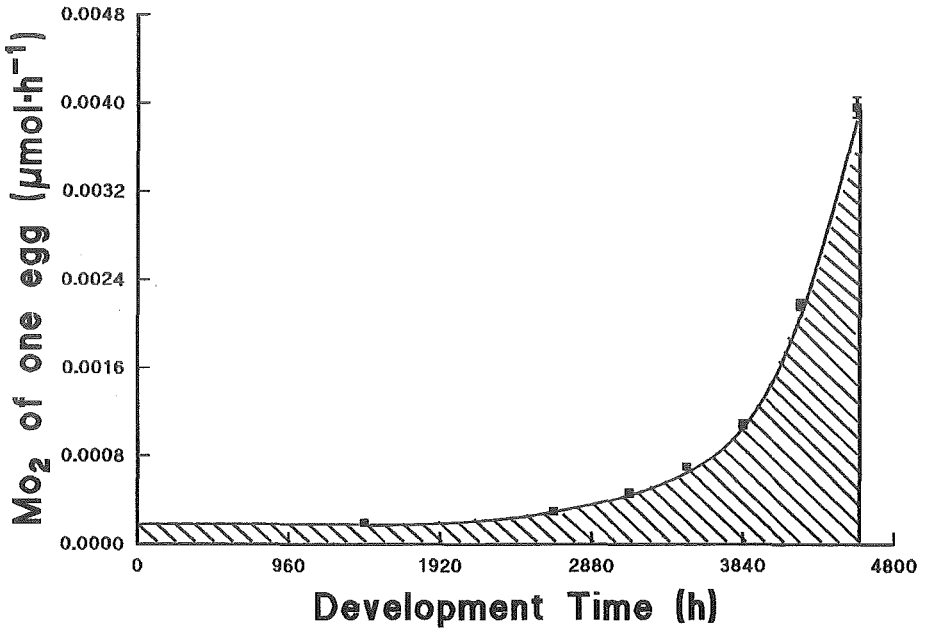


Figure 4.10 The relationships between oxygen consumption of one egg of *Heterozius rotundifrons* and development time. Each point is a mean (± SE) from 10 replicates, each of 50 eggs. Experiments were done in seawater at 15°C. Total cost of development of a single egg is 1.517 μmol O_2 (shaded area)

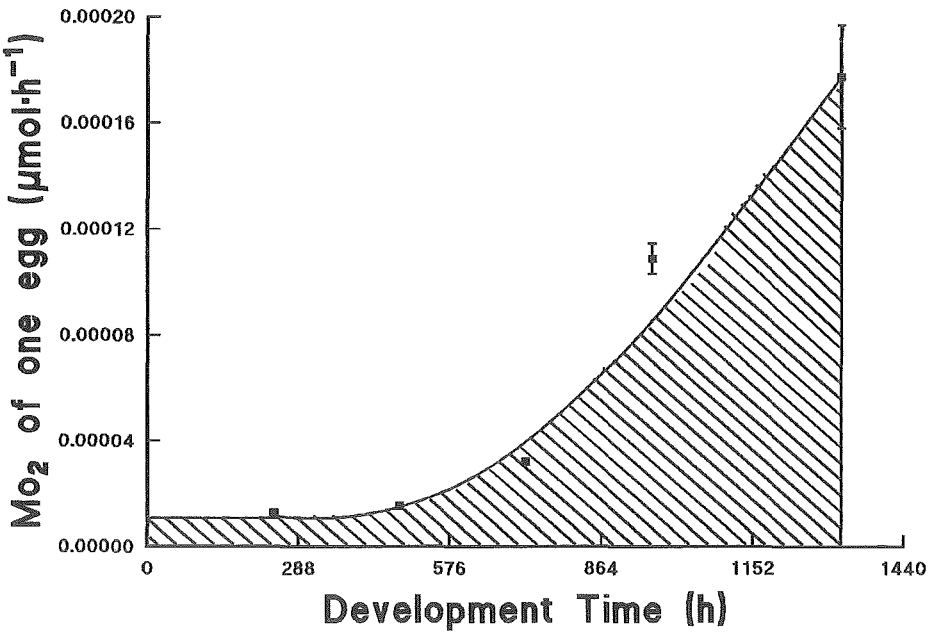


Figure 4.11 The relationships between oxygen consumption of one egg of *Cyclograpsus lavauxi* and development time. Each point is a mean (± SE) from 10 replicates, each of about 1000 eggs. Experiments were done in seawater at 15°C. Total cost of development of a single egg is 0.077 μmol O_2 (shaded area).

oxygen consumption were of similar magnitude at the start and end of development (Tables 4.4, 4.5).

Relationship of oxygen consumption between females and their embryos of H. rotundifrons and C. lavauxi.

As shown in Table 4.6, non-ovigerous crab of weight 2 g of *H. rotundifrons* and *C. lavauxi* consumed oxygen at 0.70 and 0.93 $\mu mol.g^{-1}h^{-1}$ respectively. Eggs at blastula stage of these two species consumed oxygen at 0.72 and 1.13 $\mu mol.g^{-1}h^{-1}$ which are very similar to the adult rates. However, eggs at the end of development consumed oxygen at a much greater rate per unit mass than eggs at the early stage and non-ovigerous crabs. Mass specific oxygen consumption of late stage eggs of *H. rotundifrons* and *C. lavauxi* were 9.12 and 10.81 $\mu mol.g^{-1}h^{-1}$ respectively.

Mass specific rates of oxygen consumption of the ovigerous female crabs themselves were estimated by subtracting the calculated total oxygen consumption of the whole egg mass from the oxygen consumption of ovigerous female. Mean rates of oxygen consumption of ovigerous females without their embryos (scaled to 2g bodymass) of both early and late stages of both species of crabs were slightly higher than in the corresponding non-ovigerous crabs in each case (Figure 4.6). However, a significant difference was found only between non-ovigerous and early-ovigerous (without eggs) of *H. rotundifrons* ($\dot{M}o_2$ of 2 g mass = 0.70 and 0.91 $\mu mol.g^{-1}h^{-1}$ in non-ovigerous and early-ovigerous (without eggs) respectively. There is apparently no impairment of oxygen uptake by eggs of both species in air since metabolic rates of both early and late ovigerous (without egg) are higher than non-ovigerous crabs (Table 4.6).

Table 4.6 Mass specific oxygen consumption (mean ± SE) of 2 g crabs and developing eggs of *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, in seawater and air temperature 15°C. Values of $\dot{M}o_2$ of both early and late-ovigerous (without egg) in air were obtained by substraction of the oxygen uptake by their broods in water.

Medium	Species	Status	$\dot{M}o_2$ of 2 g mass ($\mu mol.g^{-1}.h^{-1}$)	Actual mean mass (g)	n
Seawater	*H. rotundifrons*	Non-ovigerous crab	0.70 ± 0.05	2.366 ± 0.119	18
		Early-ovigerous crab	0.94 ± 0.09	2.261 ± 0.121	18
		Early-ovigerous crab (without egg)	0.91 ± 0.10	2.071 ± 0.110	18
		Early stage egg (Blastula)	0.72 ± 0.05	0.013 ± 0.000	10
		Late-ovigerous crab	1.68 ± 0.11	1.976 ± 0.223	7
		Late-ovigerous crab (without egg)	0.72 ± 0.04	1.751 ± 0.208	7
		Late stage egg (yolk 2 lobes)	9.12 ± 0.19	0.022 ± 0.000	10
	C. lavauxi	Non-ovigerous crab	0.93 ± 0.05	2.747 ± 0.181	15
		Early-ovigerous crab	1.03 ± 0.06	1.634 ± 0.139	8
		Early-ovigerous crab (without egg)	1.05 ± 0.06	1.737 ± 0.108	8
		Early stage egg (Blastula)	1.13 ± 0.09	0.009 ± 0.001	10
		Late-ovigerous crab	1.73 ± 0.10	2.448 ± 0.455	9
		Late-ovigerous crab (without egg)	1.16 ± 0.14	2.448 ± 0.516	9
		Late stage egg (yolk 2 lobes)	10.81 ± 0.63	0.023 ± 0.005	10
Air	*H. rotundifrons*	Non-ovigerous crab	0.64 ± 0.03	2.602 ± 0.184	15
		Early-ovigerous crab	0.83 ± 0.03	2.467 ± 0.209	15
		Early-ovigerous crab (without egg)	0.83 ± 0.04	2.199 ± 0.181	15
		Late-ovigerous crab	1.43 ± 0.08	2.174 ± 0.180	8
		Late-ovigerous crab (without egg)	0.71 ± 0.09	1.976 ± 0.176	8
	C. lavauxi	Non-ovigerous crab	1.83 ± 0.05	2.049 ± 0.370	11
		Early-ovigerous crab	2.08 ± 0.06	1.745 ± 0.220	5
		Early-ovigerous crab (without egg)	2.21 ± 0.08	1.530 ± 0.206	5
		Late-ovigerous crab	2.81 ± 0.06	1.578 ± 0.215	11
		Late-ovigerous crab (without egg)	2.62 ± 0.15	1.312 ± 0.173	11

DISCUSSION

Mass specific rates of oxygen consumption of inactive non-ovigerous *C. lavauxi* in air are higher than those of *H. rotundifrons*. Rates of oxygen consumption have been reported to be consistently higher in semiterrestrial than in aquatic brachyurans (Ayers, 1938; Vernberg & Vernberg, 1968; Veerannan, 1974). Hawkins *et al.* (1982) also reported that high-shore *Helice crassa* consumed more oxygen, in both air and seawater, than low-shore *Macrophthalmus hirtipes*. Ayers (1939) reported that as the habitat approaches land, there is an increase in oxygen consumption and, coincident with this, there is an increase in the activity of the crabs. The difference in the rates of oxygen consumption of two species of crabs studied here is probably also concerned with these characteristics. The high-shore *C. lavauxi* is much more active than low-shore *H. rotundifrons* in both air and seawater. *H. rotundifrons* is always found half buried under boulders where there is presumably less oxygen; it is sluggish and slow, whereas *C. lavauxi* are active and fairly swift. The partial adaptation of the respiratory mechanisms to air of *C. lavauxi* (Innes *et al.*, 1986) providing them an increased supply of oxygen, may be thought of as allowing them to have higher metabolic rates resulting in greater activity as compared to *H. rotundifrons* which always live under the rocks around the low tide area.

H. rotundifrons consumed oxygen at equal rates in air and seawater at 15°C, whereas *C. lavauxi* consumed twice as much oxygen in air. The higher rates in air suggested that *C. lavauxi* is better adapted for living in air than in water. Pearse (1929) reported that a reduction in number and volume of the gills in crabs is accompanied by an increased oxygen consumption and this indicated some sort of adaptation of the respiratory mechanism to the decreasing amounts of water and increasing amounts of air in the habitat of crabs. In *C. lavauxi*, the gill lamellae separate into regular clumps which may help to increase the surface area available for gas exchange (Innes *et al.*, 1986). Rates of oxygen consumption in air and water have been reported in a number of small New Zealand crabs and these rates were compared with the rates found in *H. rotundifrons* and *C. lavauxi* in the present study (Table 4.7). From Table 4.7, it can be seen that the metabolic rate in air of *C. lavauxi* of this study is higher than the values given by Innes *et al.* (1986). Since the experiments were done at different temperature (10°C and 15°C for Innes *et al.* (1986) and the present study respectively), the different results could be explained by an increase in metabolic rate with temperature. Rates of oxygen consumption in air were reported in other shore crabs, *Macrophthalmus hirtipes* and *Helice crassa* (Hawkins *et al.*, 1982). In these species, $\dot{M}o_2$ at 10°C were found to be

Table 4.7 A comparison of mass specific oxygen consumption of 1.5 g body mass in air and water in a number of small New Zealand shore crabs (non-ovigerous).

Species	Temperature (°C)	Rates of O_2 consumption in air ($\mu mol.g^{-1}.h^{-1}$)	Rates of O_2 consumption in water ($\mu mol.g^{-1}.h^{-1}$)	References
Cyclograpsus lavauxi	10	1.41	1.45	Innes *et al.* (1986)
Cyclograpsus lavauxi	15	1.80	0.96	Present study
Heterozius rotundifrons	15	0.73	0.73	Present study
Macrophthalmus hirtipes	10	1.33	2.30	Hawkins *et al.* (1982)
Macrophthalmus hirtipes	15	2.93	2.81	Hawkins *et al.* (1982)
Helice crassa	10	1.46	1.90	Hawkins *et al.* (1982)
Helice crassa	15	2.60	2.87	Hawkins *et al.* (1982)

similar to those of *C. lavauxi* reported by Innes *et al.* (1986) (Table 4.7). Compared with this study, at 15°C, $\dot{M}o_2$ in air of both *M. hirtipes* and *H. crassa* were higher than *C. lavauxi*. The higher rates found in both species by Hawkins *et al.* (1982) are probably not the true resting rates since the measurements were done in shaken respirometers. Rates of oxygen consumption in water at 10°C and 15°C of *M. hirtipes* and *H. crassa* were 2.30, 2.81 1.90 and 2.87 $\mu mol.g^{1}.h^{-1}$ respectively (Table 4.7). These values were very high compared with that of *C. lavauxi* (Innes *et al.*, 1986) and of *C. lavauxi* in this study. Innes, *et al.* (1986) suggested that the values found by Hawkins *et al.* could be over-estimates of the true resting rates because the settlement times of their studies were only 30 min and the respirometer flasks were shaken. The values of $\dot{M}o_2$ in water of *C. lavauxi* in this study were even lower than those found by Innes *et al.* (1986) despite the higher temperature used here. Innes *et al.* (1986) did not mention in their method whether they used a separated blank chamber or the blank chamber that connected to the animal chamber. However, in my study, I found that by using the blank chamber that connected to the animal chambers (as described in the method), gave a high value of ΔPo_2 of the blank (about one-third to one-half of ΔPo_2 of the animal chamber). Thus, Po_2 obtained from the difference between ΔPo_2 of crab and ΔPo_2 of the blank was very low and this gave a result of low $\dot{M}o_2$ in water of *C. lavauxi* in this study. This $\dot{M}o_2$ is probably near the real value of oxygen consumption of crab since $\dot{M}o_2$ of some micro-organisms which detached from the body of crab during settlement have already been subtracted from the total $\dot{M}o_2$ within the animal chamber.

Oxygen consumption measurements in both air and water of *H. rotundifrons* and *C. lavauxi* at 15°C, showed that late stage ovigerous for any weight animal, have a higher respiratory rate than non-ovigerous crabs. The difference in respiratory rates between non-ovigerous and ovigerous crabs can be taken to be due to either the increased oxygen consumed by developing eggs or the increased energy for carrying broods. However, it has been found that after substraction of the oxygen uptake by their broods, the metabolic rates of ovigerous females (both early and late stages) of these two species of crabs were similar to that of non-ovigerous crabs. However, a different significance was found in early ovigerous *H. rotundifrons*. Thus, the increased rate of oxygen consumption of ovigerous crabs must mainly be due to the respiration of their embryos. Similarly, it was reported in *Daphnia* that there was no detectable energetic cost of carrying a brood in this animal (Glazier, 1991). The differences between $\dot{M}o_2$ of ovigerous (without eggs) and non-ovigerous crabs of both species shown in Figure 4.7 suggest a small metabolic cost of bearing eggs (such as increased ventilatory activity). Glazier (1991) suggested that a brood could have a metabolic cost as it was sometimes as heavy as the female herself, but the buoyancy of *Daphia* in water probably overcomes

this problem. From my observation during collecting the specimens, I found that most ovigerous *H. rotundifrons* inhabited a lower level on the shore than non-ovigerous crabs. This is probably an adaptation of ovigerous females to sustain their use of energy as they hardly moved up to a higher level on the shoreline. In *C. lavauxi,* however, there was no distinct difference found in inhabiting area between non-ovigerous and ovigerous crabs.

In both *H. rotundifrons* and *C. lavauxi*, late-ovigerous consumed oxygen in both air and water more than early-stage ovigerous crabs. This correlated to the data on oxygen consumption of developing eggs. Eggs at early stage consumed oxygen at a lower rate than late-stage eggs. There is no impaired oxygen uptake by eggs in water of both species since metabolic rates of both the early and late ovigerous (without eggs) are higher than non-ovigerous crabs (Table 4.6). The rate of oxygen consumption in air was not measured for separated eggs. However, evidence for non-impairment of oxygen uptake by eggs in air may be inferred from similar absolute differences (ovigerous minus non-ovigerous) between air and water as shown in Figure 4.6. Since the increased metabolism of ovigerous crabs in water was due to the respiration of their embryos, the similar increase in air suggests a similar ability of breathing in air and water of developing eggs.

Oxygen consumption increased as development of eggs progressed in both species of crabs. In blastula and gastrula stages, oxygen consumption was extremely low and, on a mass specific basis, was about the same as the adult crabs, despite being lower in mass by factors of more than 10^4 (*H. rotundifrons*) and 10^6 (*C. lavauxi*). These low rates are presumably partly attributed to the relatively large proportion of the cell occupied by yolk. As development progressed, the proportion of active cytoplasm increased and respiration increased 12 and 10 times in *H. rotundifrons* and *C. lavauxi* respectively. Thus, despite differences in mass by factor of 20, the mass-specific oxygen consumptions were closely similar in the two species. Rates of oxygen consumption of eggs of these two crab species were found to be in the same range as the rates of eggs of *Carcinus maenus* (about 6.6 $\mu mol.g^{-1}.h^{-1}$ at 15°C) (Wheatly, 1981), although eggs of *C. maenus* are smaller than eggs of *H. rotundifrons* by factor of 10, and larger than eggs of *C. lavauxi* by factor of 2.

Non-ovigerous *H. rotundifrons* and *C. lavauxi* consumed oxygen at similar mass-specific rates as those found in eggs at the blastula stage, but these rates were about 10-12 times less than those found in late-stage eggs. In *Daphnia* the respiration rates of eggs and early embryos (approximately 2.0 $\mu L\ O_2\ mg^{-1}\ h^{-1}$) were only about a third of the rates

of adult females (5.25 μL O_2 mg^{-1} h^{-1}), neonates (5.96 μL O_2 mg^{-1} h^{-1}) and 3-d-old embryos (6.94 μL O_2 mg^{-1} h^{-1}) (Glazier, 1991). It has been suggested that metabolic rates of animals at rest (standard metabolism) or of animals restrained to minimal activity (routine metabolism) increase with body mass (W) at a rate of about $W^{0.75}$ (Gould, 1966; Peters, 1983 and Schmidt-Nielsen, 1984). In crustaceans, metabolic rate is also increased with body mass (Weymouth *et al.*, 1944). Table 4.8 and Figure 4.12 show the relationships between metabolic rate at 15°C and body mass of various crustaceans including non-ovigerous and developing eggs at different stages of both *H. rotundifrons* and *C. lavauxi*. It can be seen from Figure 4.12 that normal scaling of metabolic rate with body mass occurs in non-ovigerous, ovigerous and late stage eggs of both *H. rotundifrons* and *C. lavauxi* in relation to other crustaceans ($W^{0.82}$), but the relationship does not apply to developing eggs at the early stage in both species. Seymour and Bradford (1995) reported that amphibians have a low allometric exponent for the relationship between $\dot{V}o_2$ and egg size, but there is a jump in $\dot{V}o_2$ at hatching. In crab eggs of these two species, it seems that the allometric relationship can only apply to embryos which have completed organization and are ready to hatch as larvae within a few days. It is also of interest to note that in a study on ontogeny of cardiac function in crustaceans; a single power function did not apply for the relationship between heart rate and body weight of newly hatched brine shrimp (Spicer, 1994).

The metabolic cost of development, in term of oxygen consumption, of single eggs of *H. rotundifrons* and *C. lavauxi* were 1.517 and 0.077 μmol O_2 respectively. It can be seen that the total cost of development of an egg of *C. lavauxi* is about 20 times less than of *H. rotundifrons*. The 20-fold ratio is approximately equal to the ratio of their masses (about 19 times) which in turn reflects the trophic investment by the parent in each egg. The longer development time of *H. rotundifrons* than *C. lavauxi* is reflected in the slower rise in oxygen consumption in the first species. There is a correlation between the time course of changes in $\dot{M}o_2$ in eggs and the timing of disappearance of yolk (chapter 2). In both species, when the rate of oxygen consumption increased, the rate of disappearance of yolk also increased.

In conclusion, oxygen consumption measurements of both in air and water of *H. rotundifrons* and *C. lavauxi* showed that late stage ovigerous for any mass animal, have a higher respiratory rate than non-ovigerous crabs. The difference in respiratory rates between non-ovigerous and ovigerous crabs was due to the increased oxygen consumed by developing eggs since it has been found that rates of oxygen consumption of ovigerous females without their embryos of both early and late stages of these two species of crabs increased relatively little compared with non-ovigerous crabs. The small

Table 4.8 Rates of oxygen consumption in various crustaceans, non-ovigerous *Heterozius rotundifrons* and *Cyclograpsus lavauxi*, and diifferent stages of developing eggs of *H. rotundifrons* and *C. lavauxi*, in water at 15°C.

Species	Mass (g)	$\dot{M}o_2$ (μmol.h^{-1})	Reference
* *Squilla mantis*	23.0	89.8	Montuori, 1913
	6.5	22.1	Montuori, 1913
* *Callianasa subterranea*	1.5	5.74	Montuori, 1913
	0.65	2.56	Montuori, 1913
* *Carcinus maenus*	47	91.06	Montuori, 1913
	34.5	71.49	Montuori, 1913
	14	32.84	Montuori, 1913
	10	34.94	Montuori, 1913
	7	14.83	Montuori, 1913
	1.65	6.24	Montuori, 1913
* *Dromia vulgaris*	102	177.99	Montuori, 1913
	22	89.77	Montuori, 1913
* *Eriphia spinifrons*	96	153.91	Montuori, 1913
	34	83.36	Montuori, 1913
	0.45	7.53	Montuori, 1913
* *Galathea squamifera*	4	12.82	Montuori, 1913
	2.8	6.44	Montuori, 1913
* *Ilia nucleus*	7.2	12.58	Montuori, 1913
	3.5	10.06	Montuori, 1913
* *Maja verrucosa*	43	169.94	Montuori, 1913
	4.2	18.74	Montuori, 1913
	2	6.72	Montuori, 1913
* *Pachygrapsus marmoratus*	26	85.60	Montuori, 1913
	14.6	82.93	Montuori, 1913
	11.8	65.57	Montuori, 1913
	8	14.91	Montuori, 1913
	1.8	4.8	Montuori, 1913
* *Paguristes maculatus*	17.3	131.81	Montuori, 1913
	14	116.09	Montuori, 1913
	5.3	18.59	Montuori, 1913
	4.6	19.54	Montuori, 1913
* *Palinurus vulgaris*	532	1019.58	Montuori, 1913
	75	130.34	Montuori, 1913
* *Palinurus hirtellus*	3.6	12.64	Montuori, 1913
	0.24	1.61	Montuori, 1913
* *Sicyonia sculpta*	5.3	24.05	Montuori, 1913
	4.1	20.83	Montuori, 1913
	2.0	14.42	Montuori, 1913

Table 4.8 (continue)

Species	Mass (g)	$\dot{M}o_2$ (μmol.h^{-1})	Reference
* *Spirontocaris securifrons*	0.04	0.179	Fox, 1936
	0.04	0.357	Fox, 1936
* *Pandalina brevirostrus*	0.15	1.004	Fox, 1936
	0.15	0.81	Fox, 1936
* *Spirontocaris securifrons*	1.80	15.58	Fox, 1936
* *Pandalus montagui*	2.20	13.11	Fox, 1936
	2.20	12.71	Fox, 1936
* *Astacus fluviatilis*	31.0	59.09	Rogers, 1927
* *Homarus americanus*	189.0	295.31	Bosworth *et al.*, 1936
	189.0	210.94	Bosworth *et al.*, 1936
	189.0	177.19	Bosworth *et al.*, 1936
	230.0	266.80	Bosworth *et al.*, 1936
* *Homarus sp.*	315.0	960.47	Rogers, 1927
* *Homarus americanus*	324.0	409.34	Bosworth *et al.*, 1936
* *Cancer sp.*	470.0	1414.2	Rogers, 1927
* *Palinurus sp.*	520	1040	Rogers, 1927
* *Pugettia producta*	80.5	341.59	Weymouth *et al.*, 1944
** *Callinectes sapidus*	150	555	O' Mahoney, 1977
** *Carcinus maenus*	49	58.8	Taylor&Butler, 1978
** *Pagurus hirsutiusculus*	2.4	15.6	Burggren&McMahon, 1981
** *Cardisoma guanhumi* (terrestrial crab)	128	633.6	O' Mahoney, 1977
** *Gecarcinus lateralis* (terrestrial crab)	30	81	Taylor&Davies, 1982
Macrophthalmus hirtipes	1.5	4.39	Hawkins *et al.*, 1982
Helice crassa	1.5	4.30	Hawkins *et al.*, 1982
Heterozius rotundifrons (non-ovigerous)	2.366	1.64	Present study
Cyclograpsus lavauxi (non-ovigerous)	2.747	2.56	Present study
H. rotundifrons eggs (Stage 2)	0.000269	0.000195	Present study
H. rotundifrons eggs (Stage 3)	0.000286	0.000295	Present study
H. rotundifrons eggs (Stage 4)	0.000315	0.000461	Present study
H. rotundifrons eggs (Stage 5A)	0.000328	0.000701	Present study
H. rotundifrons eggs (Stage 5B)	0.000362	0.001082	Present study
H. rotundifrons eggs (Stage 5C)	0.000409	0.002170	Present study
H. rotundifrons eggs (Stage 5D)	0.000435	0.003946	Present study
C. lavauxi eggs (Stage 2)	0.0000109	0.0000124	Present study
C. lavauxi eggs (Stage 3)	0.0000120	0.0000155	Present study
C. lavauxi eggs (Stage 4)	0.0000144	0.0000321	Present study
C. lavauxi eggs (Stage 5 (A-C))	0.0000168	0.0001087	Present study
C. lavauxi eggs (Stage 5D)	0.0000203	0.0001774	Present study

* data are recalculated from Weymouth *et al.*, 1944

** data are recalculated from Burggren&McMahon, 1988

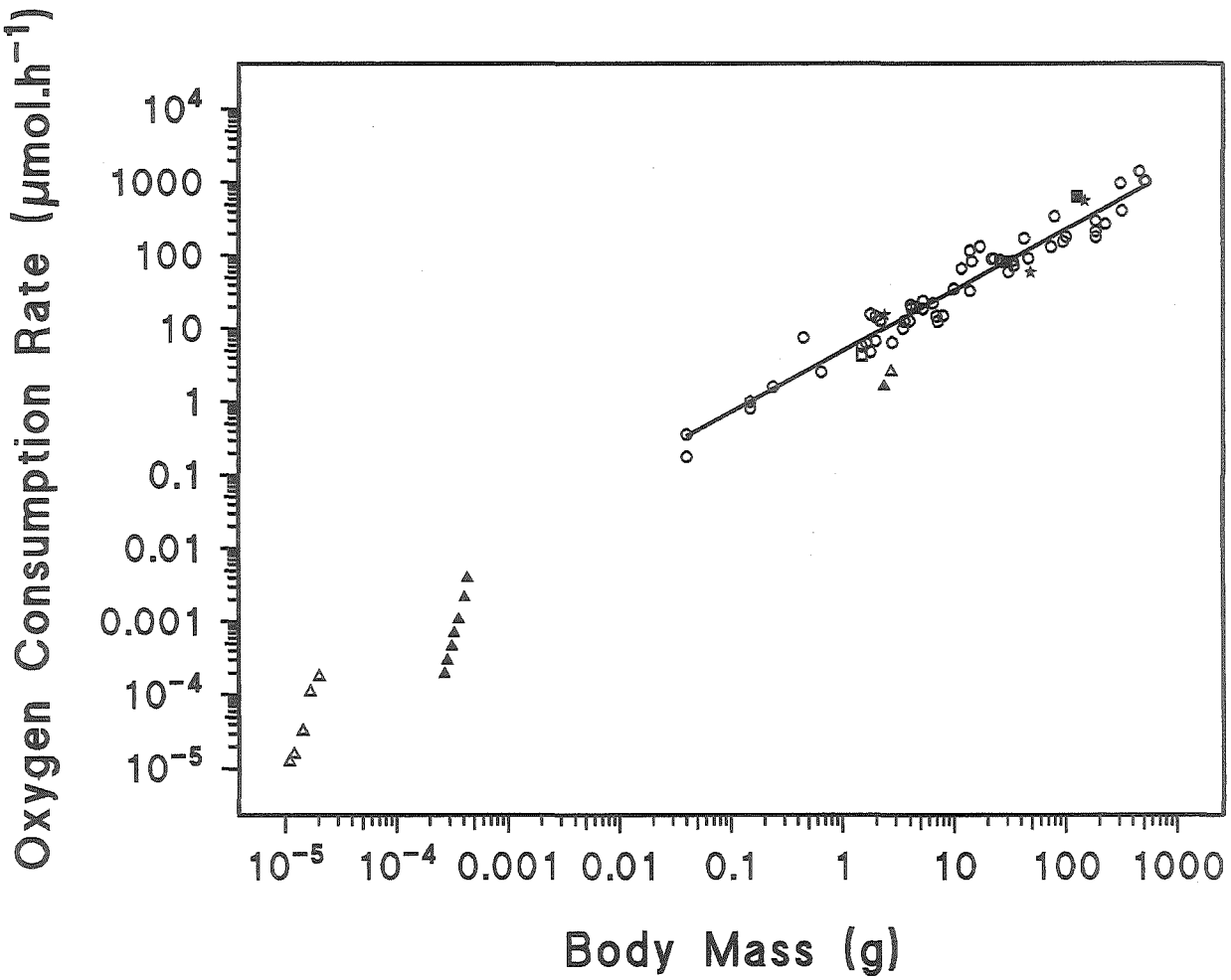


Figure 4.12 Log_{10}-transformed relationship between total oxygen consumption and body mass for various crustaceans in water (o, data were recalculated from Weymouth *et al.*, 1944, slope = 0.82; ★ and ■, data were recalculated from Burggren&McMahon, 1988; □, New Zealand intertidal crabs *Helice crassa* and *Macrophthalmus hirtipes*), for non-ovigerous crabs and developing eggs of *Heterozius rotundifrons* (▲), for non-ovigerous crabs and developing eggs of *Cyclograpsus lavauxi* (△). All data are tabulated in Table 4.8.

increase of $\dot{M}o_2$ suggested an extra component of oxygen uptake due to increased ventilation of ovigerous crabs in water. Oxygen consumption increased as development of eggs progressed in both species of crabs. Eggs at the early stage of both *H. rotundifrons* and *C. lavauxi* had mass-specific rates of oxygen consumption similar to those found in non-ovigerous crabs and these rates were about 10-12 times less than the rates found in eggs at the late stage. The normal scaling of metabolic rate with body mass can be applied to adult crabs and late stage eggs but not to the early stage eggs. The total cost of development of an egg of *C. lavauxi* is about 20 times less than that of *H. rotundifrons*. In term of an energy balance, this is an advantage since both species of crabs have similar size, thus their rates of using energy should be similar either in normal or reproductive stages. Because *C. lavauxi* produced a large number of eggs, their eggs have to be small so that the whole egg mass consumed the similar amount of energy as that consumed by the whole egg mass of *H. rotundifrons*. It is not surprising for *C. lavauxi* to produce such a large number of eggs since most marine invertebrates produced large number of eggs (Thorson, 1950). The reason for producing many small eggs in *C. lavauxi* is an advantage since many small eggs produce many small larvae and this increased the number of larvae to survive as young crabs. An alternative strategy used in *H. rotundifrons* is to produce a few large eggs. This gives a few large larvae and thus a few large young crabs. In crustaceans, larger size at settlement may improve the ability to obtain a larger array of food, to reduce competition, and to avoid predators (Mauchline, 1973 and Reaka, 1979). Larger size at settlement will also result in shorter time to sexual maturity (Hines, 1981). Thus, producing the larger eggs in *H. rotundifrons* probably gives an advantage for the survival of zoea larvae of this species if they follow the above rules. However, whether crabs produced small eggs or large eggs, the most important thing is that the total energy used by the whole egg mass should be similar for the same range of crab weight within the closely related species of crabs.

CHAPTER FIVE

RESPIRATORY PHYSIOLOGY OF DEVELOPING EGGS AND OVIGEROUS CRABS IN RESPONSE TO DECLINING OXYGEN TENSION

INTRODUCTION

The intertidal zone is characterised by extreme fluctuations in environmental variables. One of the greatest variations in the physicochemical conditions of the intertidal zone relates to "oxygen tension" or "Po_2". Decapod crustaceans occupy a wide range of habitats in the tidal areas in respect to the environmental conditions. These animals are known to have abilities in regulate their body fluid and gas tensions in response to sudden changes of the Po_2 of surrounding. Two kinds of respiratory response to hypoxia are recognised (Hill, 1976). As the environmental O_2 tensions falls animals may either experience a continuous reduction in O_2 uptake (i.e. conform to the change) or maintain O_2 uptake at the normoxic level until the O_2 tensions reach the critical point at which the O_2 uptake decreases dramatically (i.e. regulate against the change). This critical oxygen tension" or "P_{crit} " has been described by Herreid (1980) as the oxygen tension below which the rate of oxygen consumption of the animal becomes dependent upon P_IO_2 (the partial pressure of oxygen in the inspired water). Van Weel *et al.* (1954) studied seven species of shallow water crabs. They demonstrated that the rate of oxygen uptake of a mud-dwelling crab was independent of ambient oxygen tensions until a low level was reached; at this point the respiration pattern became oxygen dependent.

The P_{crit} is not a fixed point and does not identify an abrupt transition from regulating $\dot{M}o_2$ to the condition where $\dot{M}o_2$ depends on P_IO_2 (Spoek, 1974; Taylor *et al.*, 1973, 1977; Taylor, 1976; Herreid, 1980; Taylor & Wheatly, 1981; Wheatly & Taylor, 1981; Johnson & Uglow, 1987; Swain *et al.*, 1987). The value of P_{crit} is found to vary with different factors, e.g. small-sized prawns (*Penaeus indicus*) were less metabolically dependent on ambient oxygen tension below 100 mmHg than large-sized animals (Subrahmanyan, 1962). In copepod, *Calanus finmarchicus* P_{crit} values are found to be different in adult and larval stages (Marshall *et al.*, 1935). It has been reported that *Homarus gammarus* (Spoek, 1974), *Carcinus maenas* (Taylor, 1976) and *Austropotamobius pallipes* (Wheatly & Taylor, 1981) maintained their $\dot{M}o_2$ by increasing branchial ventilation as P_IO_2 declined, and when P_{crit} was reached branchial ventilation declined in all three.

The recovery of animals from varying levels of induced hypoxia has also been the subject of numerous investigations. Teal & Carey (1967), Thompson & Pritchard (1969a), McMahon & Wilkens (1972; 1975) and Taylor *et al.* (1977) have shown that a marked increase in oxygen consumption above normal resting levels occurs after exposure to hypoxia. Such elevated levels of oxygen consumption or respiratory overshoots after hypoxia have been widely reported for many decapod crustaceans and are generally considered to represent the repayment of an oxygen debt, when lactic acid which is one of the accumulated end products of anaerobic metabolism is re-oxidised (Bridges & Brand, 1980).

In non-ovigerous crabs, short-term exposure to either progressively declining Po_2 or maintained hypoxia are reported to result in an increased frequency of scaphognathite pumping and decreased heart rate (Taylor, *et al.* 1973; Taylor & Butler, 1973; Batterton and Cameron, 1978; Taylor & Wheatly, 1981). In ovigerous crabs (*Carcinus maenas*), it has been found that hypoxia results in changing the mode of ventilation of adult females. Ovigerous females were observed to reverse the direction of ventilation and direct a stream of bubbles out of the openings at the base of the posteriormost pair of walking legs and thus over the developing egg mass. On the other hand, non-ovigerous females responded to hypoxia by reversing the direction of ventilation and directing a stream of bubbles through the large Milne-Edwards openings at the base of the chelae. The P_IO_2 at which the behaviour is first observed coincided with the critical oxygen tension below which oxygen consumption of their developing eggs became dependent on further reduction in Po_2 and this P_IO_2 was significantly higher than in non-ovigerous crabs (Wheatly, 1981).

The New Zealand "Big-handed Crab" *Heterozius rotundifrons* (Belliidae) and the "Smooth Shore Crab" *Cyclograpsus lavauxi* (Grapsidae) are commonly found inhabiting the intertidal zone along the coastline of New Zealand. Both species occupy different positions of the intertidal zone (low-shore and high-shore respectively). Ovigerous *H. rotundifrons* spend most of their time half-buried in the moist sand where low oxygen tensions are expected during the period of low tide. It is therefore pertinent to enquire whether ovigerous females or their developing eggs have any special mechanism which allow them to survive exposure to hypoxic water? *C. lavauxi,* probably do not experience hypoxic conditions since they spend most of their time out of seawater. It is thus of interest to compare exposure to hypoxia in these two crab species.

Except for the work done by Wheatly (1981) mentioned above, until now there is still no report of work done on the effect of hypoxia on respiration of developing eggs and ovigerous crabs. The question about whether developing eggs of other intertidal crabs are conformers or regulators with respect to its oxygen uptake as environmental oxygen tension falls, is thus of interest. Since crab eggs have no ventilatory and circulatory systems, what is the compensatory mechanisms regulating their oxygen consumption? Adult *Carcinus maenas* (Portunidae) exhibits a number of ventilatory adaptations to shore life (reversed ventilation, the "emersion response") (Wheatly, 1981) which may not be typical of intertidal crabs. Thus it is also of interest to examine the patterns of respiratory behaviour in response to hypoxia in both ovigerous and non-ovigerous individuals of shore crabs from different families. The purpose of the present investigation was, therefore, to examine the effects of changes in Po_2 on heart beat and scaphognathite activity of non-ovigerous and ovigerous crabs *H. rotundifrons* (Belliidae). Comparative investigations on the effects of changes in Po_2 on oxygen consumption between developing eggs of *H. rotundifrons* and *C. lavauxi* were also investigated. Finally, the accumulation of lactic acid during hypoxia of developing eggs of *H. rotundifrons* was also measured.

MATERIALS AND METHODS

Oxygen consumption of crab eggs during progressive hypoxia at 15°C

Oxygen consumption during progressive hypoxia of eggs at stage 5D of *H. rotundifrons* and *C. lavauxi* was measured in a respiration cell of volume 1 mL, using an oxygen electrode connected to an oxygen meter (Strathkelvin Instruments 781b). The oxygen electrode was calibrated in a zero Po_2 solution and Po_2 of ambient air at water bath temperature 15°C using humidified air. The respiration cell was equipped with a small magnetic stirrer to prevent stratification of the oxygen concentration.

Crab eggs were placed into the respiration cell and left to adjust to the experimental temperature for 5 min. The rate of O_2 depletion was then recorded as the oxygen tension of the seawater in the respirometer declined from 135 mmHg (= 17.95 kPa) down to 7.5 mmHg (= 0.1 kPa), the time course of this depletion being dependent upon the amount of eggs put into the respirometer. In this study, 50 eggs of *H. rotundifrons* (0.0201 to 0.0206 g) or 883-1147 eggs of *C. lavauxi* (0.0185 to 0.0240 g) were used for each series of measurements. The approximate time for each run was 90 and 60 min for *H. rotundifrons* and *C. lavauxi* respectively.

Egg lactate determination in eggs of H. rotundifrons

Total lactate content was determined for normoxic and hypoxic eggs at the stage 5D of *H. rotundifrons*. Mean egg volume was estimated for a batch of eggs before the measurements. A group of 120 eggs was placed in the same respiration cell used for the previous hypoxia experiment. The test eggs were left to consume oxygen in the respiration cell until the Po_2 in the respiration cell was reduced to 20 mmHg (taking about 30 min). The test eggs were then subjected to a further sustained hypoxic period of 0.5, 1, 1.5, 2 and 3 hours. Eggs were then removed for lactate determination. For control normoxic egg lactate determination, another group of 120 eggs from the same batch was subjected to continued aeration and lactate determined using the same method. All experiments were carried out at 15°C.

Lactate measurement was determined in 50 µL samples. Four subsample of hypoxic eggs were derived from 120 homogenized eggs deproteinated with 240 µL of 1 molar perchloric acid and neutralised with 24 µL of 5 molar K_2CO_3. The protein precipitate

thus formed was centrifuged to the bottom of an Eppendorf tube, and the supernatant was transferred to another Eppendorf tube. This supernatant was then centrifuged again and a clear supernatant was then subdivided into four subsamples for lactate determination. Lactic acid in each subsamples was determined immediately using a Boehringer-Mannheim lactate test kit (Boehringer kit #139084). Changes in the absorbances of the samples were read at 340 nm with a Kontron Uvikon 860 spectrophotometer. The interferences due to coloured material in the eggs was eliminated using blanks prepared from homogenised eggs. The accuracy of the method was checked by standard addition.

Recording of heart and scaphognathite activity of non-ovigerous and ovigerous H. rotundifrons in response to declining oxygen tension

Measurements of heart and ventilation frequency were obtained in both non-ovigerous and ovigerous crabs. After implanting electrode and cannulae (see below), crabs were left overnight to recover in a small rectangular jar, 5 cm wide × 12 cm long × 12 cm high filled to a depth of 0.5 cm with gravel and covered to a depth of 5 cm with well-aerated seawater at 20±1 °C. The sides of the jar were covered with black paper to minimize visual disturbance to the animal. Recording wires and polythene tubing were of sufficient length to allow the crab freedom of movement within the jar. After the overnight recovery, the crab was allowed to take up a resting position in well-aerated seawater in the jar for one hour prior to the onset of hypoxia. This provided a period during which observations of behaviour in normoxia could be made for comparison with the changing patterns of activity during progressive hypoxia. The measurement on hypoxia was then made with progressive reduction in oxygen tension from settled normoxic level (Po_2 = 150 mmHg) down to approximately 15 mmHg. The progressive reduction oxygen tension of seawater was obtained using a gas mixing flow meter (Cameron instrument Co. GF-3) in which the flow rates of nitrogen and oxygen were regulated at a rate of 200 mL.min^{-1}. Four successively lower levels of oxygen tension (100, 50, 30 and 15 mmHg) were introduced into the chamber for 12 minutes each. After exposure to the lowest level of oxygen tension, the crab was allowed to ventilate in well-aerated seawater again for 10 and 30 minutes. During these periods of time, the heart and scaphognathite frequency was simultaneously monitored for 2 minutes sample period in each level of oxygen tension of seawater and after the recovery period in normoxic seawater. The behaviours of crabs during the progressive hypoxia and after the recovery period were also observed simultaneously during the measurements.

Heart beating was recorded by means of an impedance technique (Hoggarth and Trueman, 1967). Two fine silver wire electrodes were inserted through holes drilled on either side of the heart and held firmly in place with cyanoacrylate glue and rubber dam. These wires were then connected to an impedance coupler (Strathkelvin, Bioscience #A100 power supply), and the signals displayed and recorded through a recorder (Kipp & Zonen)

Fluctuations in hydrostatic pressure in the branchial cavities caused by movement of the scaphognathites were recorded using a pressure transducer (Bell & Howell, 4-327 blood pressure transducer). One branchial chamber was cannulated with Portex polythene tubing (ID 0.86 mm, OD 1.52 mm) through a hole drilled in the posterior margin of the carapace overlying gills 7 and 8. The observed pressure fluctuations were amplified by Gould 13-4615-58 universal amplifier) and displayed on Gould recorder 2200S. The results indicated both frequency and mode (forward or reverse) of scaphognathite beating, although the frequency was estimated more easily using the impedance technique similar to that used for heart rate.

To monitor scaphognathite frequency, two fine silver electrodes were inserted through holes drilled in the carapace at locations over the dorsal and ventral sides of the scaphognathites and held firmly in place with cyanoacrylate glue and rubber dam. These wires were then connected to the same impedance coupler and the signals from movement of scaphognathite were displayed through the same recorder used above for heart beat. This method of measurement gave a clearer record of scaphognathite rate and confirmed the correlation between the branchial pressure waveform and the movement of the scaphognathite but did not indicate the direction of pumping.

RESULTS

Oxygen consumption of eggs of H. rotundifrons and C. lavauxi during declining ambient oxygen tension

Mass specific oxygen consumption (mean ± S.E.) of developing eggs at stage 5D of *H. rotundifrons* and *C. lavauxi* were measured at declining oxygen tensions and were plotted against the Po_2 surrounding the eggs inside the respiration cell at 15°C (Figure 5.1, Table 5.1). For *H. rotundifrons*, eggs showed a high rate of oxygen consumption at normal oxygen tensions, but this declined progressively as the Po_2 of the water was reduced, i.e. under these conditions the eggs exhibited a low level of respiratory independence. In contrast, oxygen consumption of eggs of *C. lavauxi* was decreased by only 30% down to about 45 mmHg below which the oxygen consumption of the eggs was strongly attenuated by further reduction in Po_2. (Figure 5.1, Table 5.2).

Lactate concentration in eggs of H. rotundifrons

The effect of hypoxia on lactate accumulation in eggs of *H. rotundifrons* was examined during a period of 0.5, 1, 1.5, 2 and 3 hours. It was found that the 3 hours exposure of eggs to hypoxic seawater had a marked effect on the whole egg lactate concentration (Figure 5.2, Table 5.3). The lactate concentration of eggs isolated in normoxic seawater for up to three hours was 0.602 $mmol.L^{-1}$. During hypoxia, the concentration increased linearly to about 10 $mmol.L^{-1}$ after three hours. From the regression, the mean rate of lactate formation was 3.43 $mmol.L^{-1}.h^{-1}$ (or 3.057 $\mu mol.g^{-1}.h^{-1}$).

General behaviour of non-ovigerous and ovigerous H. rotundifrons in response to reduced ambient oxygen tension

In aerated seawater, both non-ovigerous and ovigerous crabs showed similar behaviours. Crabs usually took up resting positions by sitting quietly on the sand with their legs flexed beneath them. The chelipeds were lowered. For the majority of the time the third maxillipeds were held close to the body, covering the other mouthparts; at intervals, however they were moved vigorously from side to side. Recordings of the pressure changes in the branchial chambers which are described below, revealed that these movements of the maxillipeds were associated with periods of reversed ventilation.

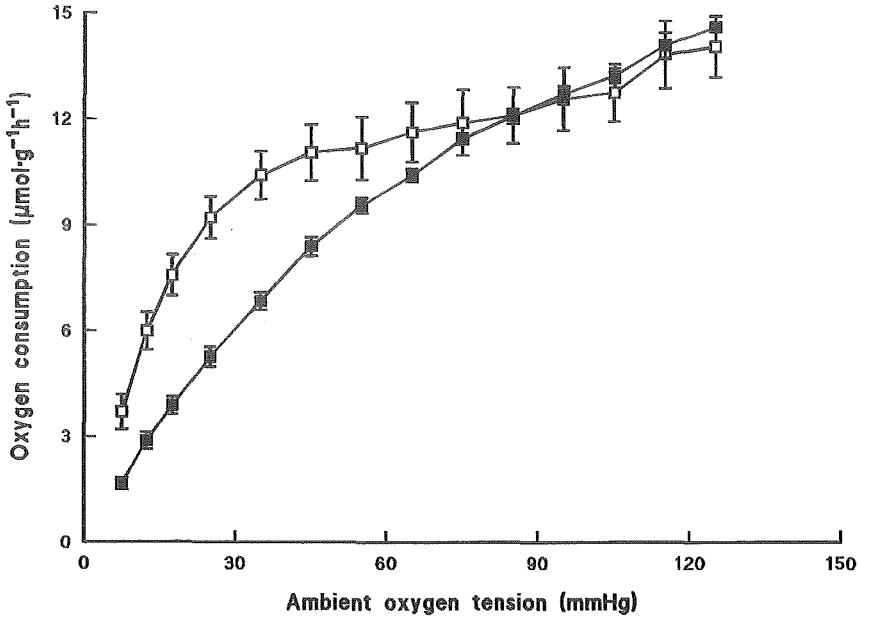


Figure 5.1 Mass specific oxygen consumption during declining ambient oxygen tension of developing eggs at stage 5D of *Heterozius rotundifrons* (■) and *Cyclograpsus lavauxi* (□) in seawater at 15°C. The point represent mean ± SE (n=4).

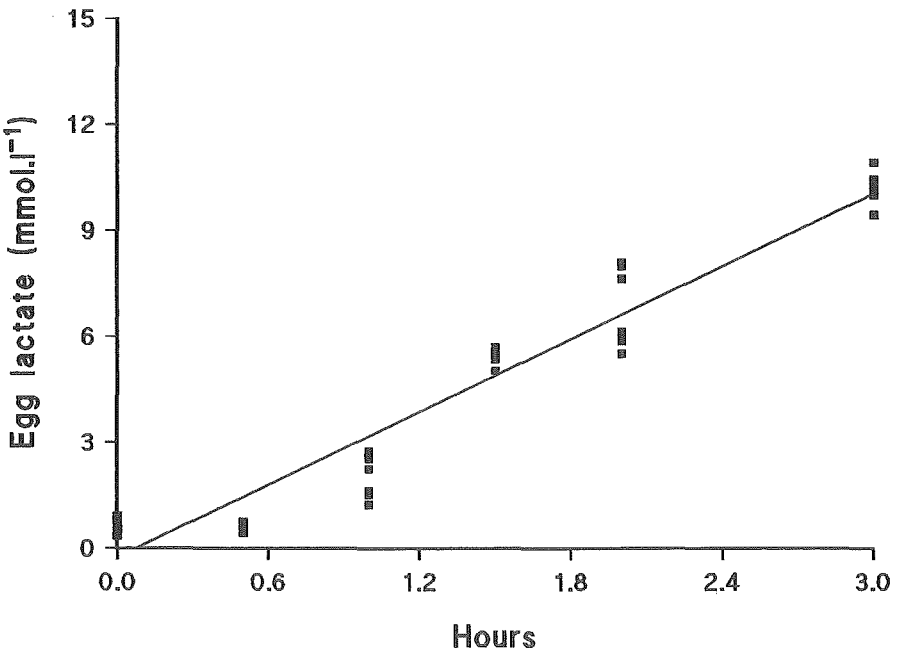


Figure 5.2 Relationship between whole egg lactate concentration at stage 5D of *Heterozius rotundifrons* and time after exposure to hypoxic water at 15°C. The regression line is Y = 3.4323 (x) - 0.264441, r = 0.96956.

Table 5.1 Mass specific oxygen consumption during hypoxia of eggs of *Heterozius rotundifrons* at stage 5D (yolk 2 lobes) in seawater at temperature 15°C. Each replicate series presents a group of 50 eggs in a 1 mL respiration cell for a period of approximately 90 min. Four different egg batches were used for four different replicates of eggs.

Po_2 (mmHg)	Mass specific oxygen consumption of developing eggs ($\mu mol.g^{-1}h^{-1}$)				
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean ± SE
135	16.14	-	15.84	13.21	15.1 ± 0.7
125	15.05	13.83	15.17	14.38	14.6 ± 0.3
115	13.45	13.32	14.86	14.75	14.1 ± 0.4
105	12.80	12.85	13.6	13.69	13.2 ± 0.2
95	12.80	12.00	12.87	13.21	12.7 ± 0.2
85	11.57	12.00	12.01	12.76	12.1 ± 0.2
75	10.92	11.53	11.62	11.71	11.4 ± 0.1
65	10.52	10.52	9.80	10.82	10.4 ± 0.2
55	9.13	10.09	9.12	9.88	9.6 ± 0.2
45	8.32	9.02	7.59	8.60	8.4 ± 0.3
35	6.65	7.60	6.24	6.84	6.8 ± 0.2
25	5.27	6.13	4.59	5.05	5.3 ± 0.3
17.5	3.83	4.68	3.40	3.59	3.9 ± 0.2
12.5	2.69	3.67	2.39	2.77	2.9 ± 0.2
7.5	1.48	2.23	1.33	1.58	1.7 ± 0.2

Table 5.2 Mass specific oxygen consumption during hypoxia of eggs of *Cyclograpsus lavauxi* at stage 5D (yolk 2 lobes) in seawater at temperature 15°C. Each replicate series presents a group of approximate 1000 eggs in a 1 mL respiration cell for a period of approximately 60 min. Four different egg batches were used for four different replicates of eggs.

Po_2 (mmHg)	Mass specific oxygen consumption of developing eggs ($\mu mol.g^{-1}h^{-1}$)				
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Mean ± SE
135	20.49	11.65	15.79	14.11	16.8 ± 1.6
125	14.96	11.20	15.79	14.28	14.1 ± 0.9
115	15.17	10.86	15.79	13.49	13.8 ± 0.9
105	13.49	10.11	14.49	12.91	12.7 ± 0.8
95	14.28	9.77	13.99	12.26	12.6 ± 0.9
85	13.26	9.77	13.84	11.56	12.1 ± 0.8
75	13.72	9.04	13.39	11.45	11.9 ± 0.9
65	13.54	9.16	12.70	11.14	11.6 ± 0.8
55	13.09	8.88	12.70	10.03	11.2 ± 0.9
45	12.47	8.61	12.44	10.65	11.0 ± 0.8
35	11.56	8.51	11.84	9.71	10.4 ± 0.7
25	9.34	7.54	10.90	9.06	9.2 ± 0.6
17.5	6.89	6.44	9.47	7.49	7.6 ± 0.6
12.5	5.14	5.05	7.70	6.07	6.0 ± 0.5
7.5	2.49	3.22	5.17	3.91	3.7 ± 0.5

Table 5.3 Lactate concentration in normoxic and hypoxic eggs (stage 5D) of *H. rotundifrons* at different period of time, temperature 15°C

Sample	Lactate concentration in eggs ($mmol.L^{-1}$)								
	30 minutes		1 hour		1.5 hours	2 hours		3 hours	
	Egg batch No. 1	Egg batch No. 2	Egg batch No. 3	Egg batch No. 4	Egg batch No. 5	Egg batch No. 6	Egg batch No. 7	Egg batch No. 8	Egg batch No. 9
Normoxic eggs	0.617	0.438	0.345	0.629	0.458	0.578	0.774	0.674	0.908
Hypoxic eggs									
Replicate 1	0.431	0.625	1.575	2.54	5.02	7.62	5.5	9.44	9.99
Replicate 2	0.698	0.704	1.235	2.24	5.49	7.99	6.12	10.23	10.92
Replicate 3	-	0.757	1.615	2.72	5.36	7.62	5.85	10.23	10.44
Replicate 4	-	-	1.515	2.75	5.68	8.08	6.01	10.17	10.23

Upon exposure to progressive hypoxia, when ambient Po_2 was about 30 mmHg, the crabs immediately became agitated and frequently stood with legs extended and ventral surface of the abdomen elevated off the floor of the chamber. Elevation activity ceased within a few minutes, and the animals then became quiescence for the duration of the hypoxic exposure. During this quiescent period the crabs no longer responded to visual stimuli. Infrequently the crabs would resume the intermittent sporadic activity for 20-30 seconds and then return to quiescence. Eventually the crabs' antennule and mouthpart movement ceased, at this stage, the mouthparts of crabs became slightly opened, however they failed to respond to any type of stimulus. Crabs were able to stay in this hypoxic condition for at least half an hour. When the oxygen tension of seawater was increased rapidly by aeration, the crabs immediately resumed antennule and mouthpart movements.

Heart rates and scaphognathite activities of non-ovigerous and ovigerous crabs in aerated seawater

In aerated seawater, the hearts of both non-ovigerous and ovigerous crabs exhibit long periods of steady beating interspersed with periods of arrhythmia. Heart rates sometimes show an arrhythmia including periods of low frequency and increased amplitude which occurred in association with the changes in frequency beating of scaphognathite (Figure 5.3). Mean heart rate of non-ovigerous and ovigerous crabs in normoxic seawater were similar (189±7 and 176±10 beats.min^{-1} respectively, Table 5.4).

Both non-ovigerous and ovigerous crabs showed continuous scaphognathite beating with regular switches from a forward to reversed direction. Mean forward ventilation frequency (one side only) of non-ovigerous and ovigerous crabs were 233±20 and 249±38 beats.min^{-1} respectively and varied between 195 to 264 beats.min^{-1} in non-ovigerous crab and between 153 to 369 beats.min^{-1} in ovigerous crab (Table 5.5) with the very rapid rates of scaphognathite beating occurring during and sometimes immediately after reversed ventilation. Both non-ovigerous and ovigerous crabs ventilated predominantly in the forward mode. Reversed ventilation was less frequent (about 20% and 10% of the total time in non-ovigerous and ovigerous crabs respectively) (Table 5.7). Reversed ventilation frequency in non-ovigerous and ovigerous crabs were 267±13 and 270 beats.min^{-1} respectively (Table 5.6). No ventilatory pauses were observed in crabs in normoxic seawater.

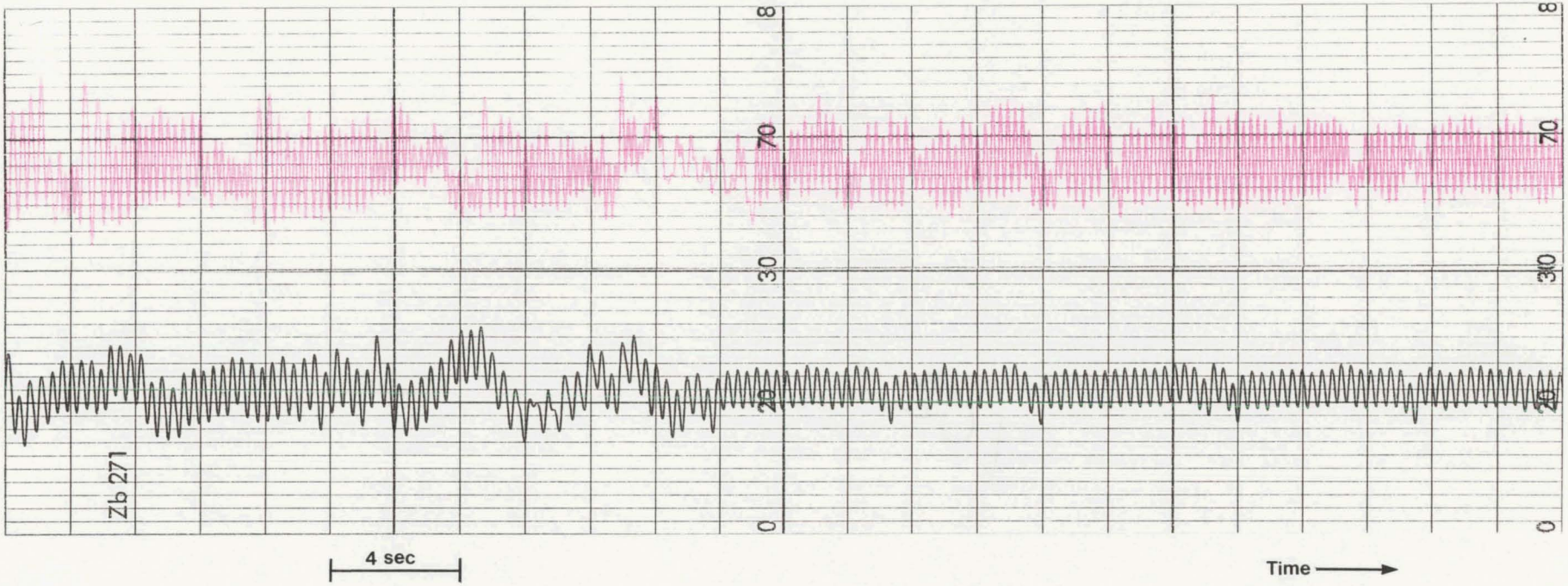


Figure 5.3 Pattern of heart rate and ventilation of ovigerous *H. rotundifrons* in normoxic seawater recorded by means of an impedance technique. Black line: heart rates; Red line: ventilation rates. Heart rates sometimes show an arrhythmia including periods of low frequency and increased amplitude which occurred in association with the changes in beat frequency of scaphognathite.

Table 5.4 Value and mean value (± SE) of heart rates (beats.min^{-1}) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

Po_2 (mmHg)	Heart rate (beats.min^{-1})									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	183	181	202	189 ±7	202	159	151	195	175	176 ± 10
100	181	114	180	158 ± 22	172	129	129	153	150	146 ± 8
50	137	91	120	116 ± 13	142	81	103	126	112	113 ± 10
30	90	69	60	73 ± 9	94	55	81	67	55	70 ± 8
15	52	29	34	38 ± 7	42	22	33	24	5	25 ± 6
After 10 min recovery period	184	196	213	198 ± 8	216	180	152	213	165	185 ± 13
After 30 min recovery period	192	201	219	204 ± 8	216	183	192	222	159	194 ± 11

Table 5.5 Value and mean value (± SE) of forward ventilation frequency (beats.min^{-1}) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

Po_2 (mmHg)	Forward ventilation frequency (beats.min^{-1})									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	195	264	240	233 ±20	277	181	153	264	369	249 ± 38
100	322	192	195	236 ± 43	236	256	166	no vent.	354	253 ± 39
50	236	149	135	173 ± 32	331	154	264	no vent.	243	248 ± 37
30	208	58	81	116 ± 47	162	118	274	no vent.	148	175 ± 34
15	124	18	-	71 ± 53	49	no vent.	91	no vent.	no vent.	70 ± 21
After 10 min recovery period	280	306	213	266 ± 28	334	297	184	348	290	291 ± 29
After 30 min recovery period	270	312	219	267 ± 27	283	348	279	360	249	304 ± 21

Table 5.6 Value and mean value (± SE) of reversed ventilation frequency (beats.min^{-1}) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

Po_2 (mmHg)	Reversed ventilation frequency (beats.min^{-1})									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	286	273	242	267 ± 13	-	270	-	-	-	270 ± 0
100	369	-	250	309 ± 59	-	264	-	no vent.	-	264 ± 0
50	222	228	-	225 ± 3	-	-	-	no vent.	-	-
30	-	-	90	90 ± 0	-	-	-	no vent.	195	195 ± 0
15	157	180	55	131 ± 38	-	no vent.	-	no vent.	no vent.	-
After 10 min recovery period	330	-	195	262 ± 67	-	-	249	-	207	228 ± 21
After 30 min recovery period	228	310	-	269 ± 41	-	225	-	201	-	213 ± 12

Table 5.7 Value and mean value (± SE) of time spent reversed ventilation (%) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

Po_2 (mmHg)	Time spent reversed ventilation (%)									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	13.3	20.9	30.3	21.5 ± 4.9	12.1	11.3	3.1	0.6	25.0	10.4 ± 4.3
100	21.7	2.2	7.2	10.3 ± 5.9	4.4	13.3	0	0	6.2	4.8 ± 2.4
50	15.4	10.9	0.4	8.9 ± 4.5	4.8	2.2	1.8	0	14.5	4.6 ± 2.6
30	10.1	4.0	7.3	7.1 ± 1.8	6.4	11.5	5.0	0	88.4	22.3 ± 16.6
15	11.1	8.6	63.8	27.8 ± 18.0	3.9	4.0	1.8	8.0	4.3	4.4 ± 1.0
After 10 min recovery period	15.3	44.5	43.3	34.4 ± 9.5	17.1	17.1	12.3	0.6	75.3	24.5 ± 13.1
After 30 min recovery period	15.1	23.7	12.1	17.0 ± 3.5	12.0	21.6	7.9	14.8	8.3	12.9 ± 2.5

During forward pumping, the scaphognathite produced a negative pressure in the branchial cavity relative to that outside of the animal. Mean negative pressure of non-ovigerous and ovigerous crabs in normoxic seawater were -5.7±2.7 mmH_2O (varied between -3.0 to -11.0 mmH_2O) and -5.2±1.0 mmH_2O (varied between -3.0 to -9.0 mmH_2O) respectively (Table 5.8). A reversal of the scaphognathite produced a positive pressure which the mean values were 7.0±1.2 mmH_2O (varied between 5.0 to 10.0 mmH_2O) and 6.7±1.2 mmH_2O (varied between 5.0 to 9.0 mmH_2O) in non-ovigerous and ovigerous crabs respectively (Table 5.9).

The overall patterns of heart and scaphognathite activity from both non-ovigerous and ovigerous crabs recorded while the animals were in buried and unburied conditions were compared. There was no obvious or consistent differences in heart rates and scaphognathite activity in the different conditions or between non-ovigerous and ovigerous crabs (Table 5.10).

Effect of reduced oxygen tension on heart rates and scaphognathite activities

Both non-ovigerous and ovigerous crabs showed a trend of decreased heart rate associated with the reduced oxygen tension. Figure 5.4 illustrates the changes in the heart rates during progressive hypoxia and after recovery periods. The heart rates decreased from 189±7 $beats.min^{-1}$ in normoxic seawater to 38±7 $beats.min^{-1}$ in seawater at Po_2 15 mmHg and from 176±10 $beats.min^{-1}$ in normoxic seawater to 25±6 $beats.min^{-1}$ in seawater at Po_2 15 mmHg in non-ovigerous and ovigerous crabs respectively. Following aeration to seawater, in which crabs had been exposed to very low oxygen tensions for a period, the heart rate of crabs increased rapidly beyond the normal level only after 10 min of aeration and it was still higher than the normal rates after 30 min of recovery period. (Table 5.4). There was no significant difference between heart rates of non-ovigerous and ovigerous crabs in response to each reduced oxygen tension.

The general response of scaphognathites to oxygen deprivation differed from that described for heart rates. In both non-ovigerous and ovigerous crabs, both forward and reversed ventilation did not decrease until Po_2 reduced to 50 mmHg. At Po_2 about 30 mmHg, ventilatory activity became very slow and sometimes ceased. Upon being returned to normoxic seawater, ventilation rates increased to more than its original normoxic levels (Table 5.5 and Figure 5.4).

Table 5.8 Value and mean value (± SE) of forward branchial pressure (mm H_2O) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

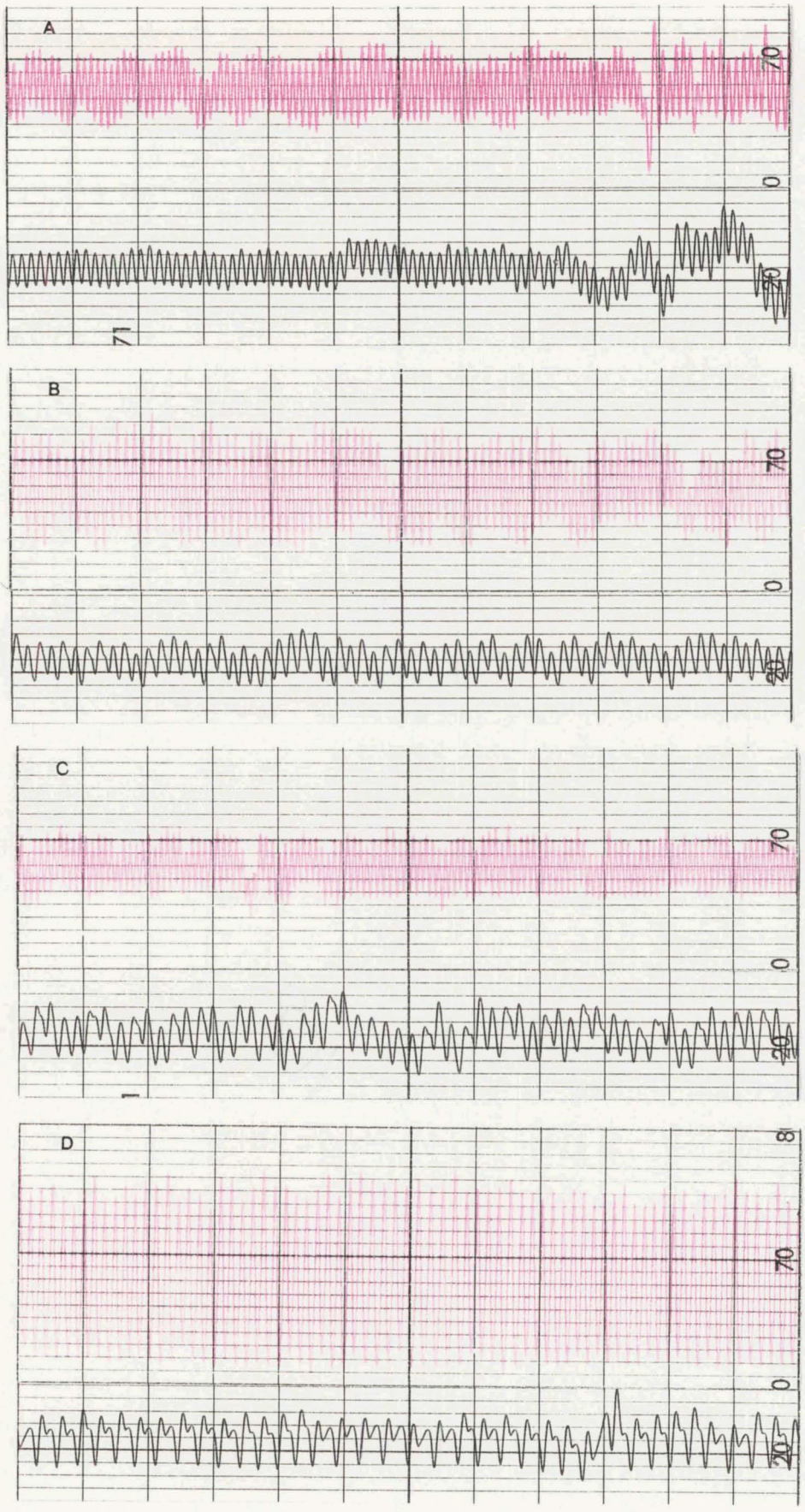
Po_2 (mmHg)	Forward branchial pressure (mm H_2O)									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	-3.0	-3.0	-11.0	-5.7 ± 2.7	-9.0	-5.0	-3.0	-4.0	-5.0	-5.2 ± 1.0
100	-5.0	-4.0	-12.0	-7.0 ± 2.5	-8.0	-4.0	-2.0	no vent.	-4.0	-4.5 ± 1.3
50	-10.0	-8.0	-5.0	-7.7 ± 1.4	-6.0	-3.0	-2.0	no vent.	-4.0	-3.7 ± 0.8
30	-5.0	-5.0	-4.0	-4.7 ± 0.3	-6.0	-3.0	-3.0	no vent.	-4.0	-4.0 ± 0.7
15	-3.0	-6.0	-	-4.5 ± 1.5	-5.0	no vent.	-2.0	no vent.	no vent.	-3.5 ± 1.5
After 10 min recovery period	-6.0	-6.0	-6.0	-6.0 ± 0.0	-15.0	-8.0	-3.0	-6.0	-5.0	-7.4 ± 2.1
After 30 min recovery period	-3.5	-6.0	-10.0	-6.5 ± 1.9	-9.0	-5.0	-10.0	-8.0	-6.0	-7.6 ± 0.9

Table 5.9 Value and mean value (± SE) of reversed branchial pressure (mm H_2O) of non-ovigerous and ovigerous *H. rotundifrons* during progressive hypoxia and subsequent normoxic recovery, temperature 20 ± 1°C.

Po_2 (mmHg)	Reversed branchial pressure (mm H_2O)									
	Non-ovigerous				Ovigerous					
	#1	#2	#3	Mean ± SE	#1	#2	#3	#4	#5	Mean ± SE
150	5.0	6.0	10.0	7.0 ± 1.2	9.0	5.0	-	-	6.0	6.7 ± 1.2
100	6.0	-	14.0	10.0 ± 4.0	1.3	7.0	-	no vent.	-	10.0 ± 3.0
50	11.0	11.0	-	11.0 ± 0.0	-	-	-	no vent	-	-
30	-	-	5.0	5.0	-	-	-	no vent	2.0	2.0
15	4.0	6.0	3.0	4.3 ± 0.9	-	no vent.	-	no vent	no vent.	-
After 10 min recovery period	7.0	-	8.0	7.5 ± 0.5	1.4	8.0	10.0	6.0	5.0	8.6 ± 1.6
After 30 min recovery period	5.0	7.0	10.0	7.3 ± 1.4	1.4	5.0	-	11.0	8.0	9.5 ± 1.9

Table 5.10 Heart rates, forward ventilation frequency and time spent forward ventilation of unburied and buried of non-ovigerous and ovigerous *H. rotundifrons* during exposure in normal air, temperature 20 ± 1°C.

Crab	Heart rate (beats.min^{-1})	Forward ventilation frequency (beats.min^{-1})	Time spent forward ventilation (%)
Unburied			
Non-ovigerous #1	183	195	86.7
Non-ovigerous #2	181	264	79.0
Non-ovigerous #3	202	240	69.7
Ovigerous #1	202	277	87.9
Ovigerous #2	159	181	88.7
Ovigerous #3	151	153	96.9
Ovigerous #4	195	264	99.3
Ovigerous #5	175	369	74.9
Buried			
Non-ovigerous #1	189	259	80.0
Non-ovigerous #2	135	144	91.2
Ovigerous #1	180	225	80.0
Ovigerous #2	153	182	78.4
Ovigerous #3	156	213	91.2



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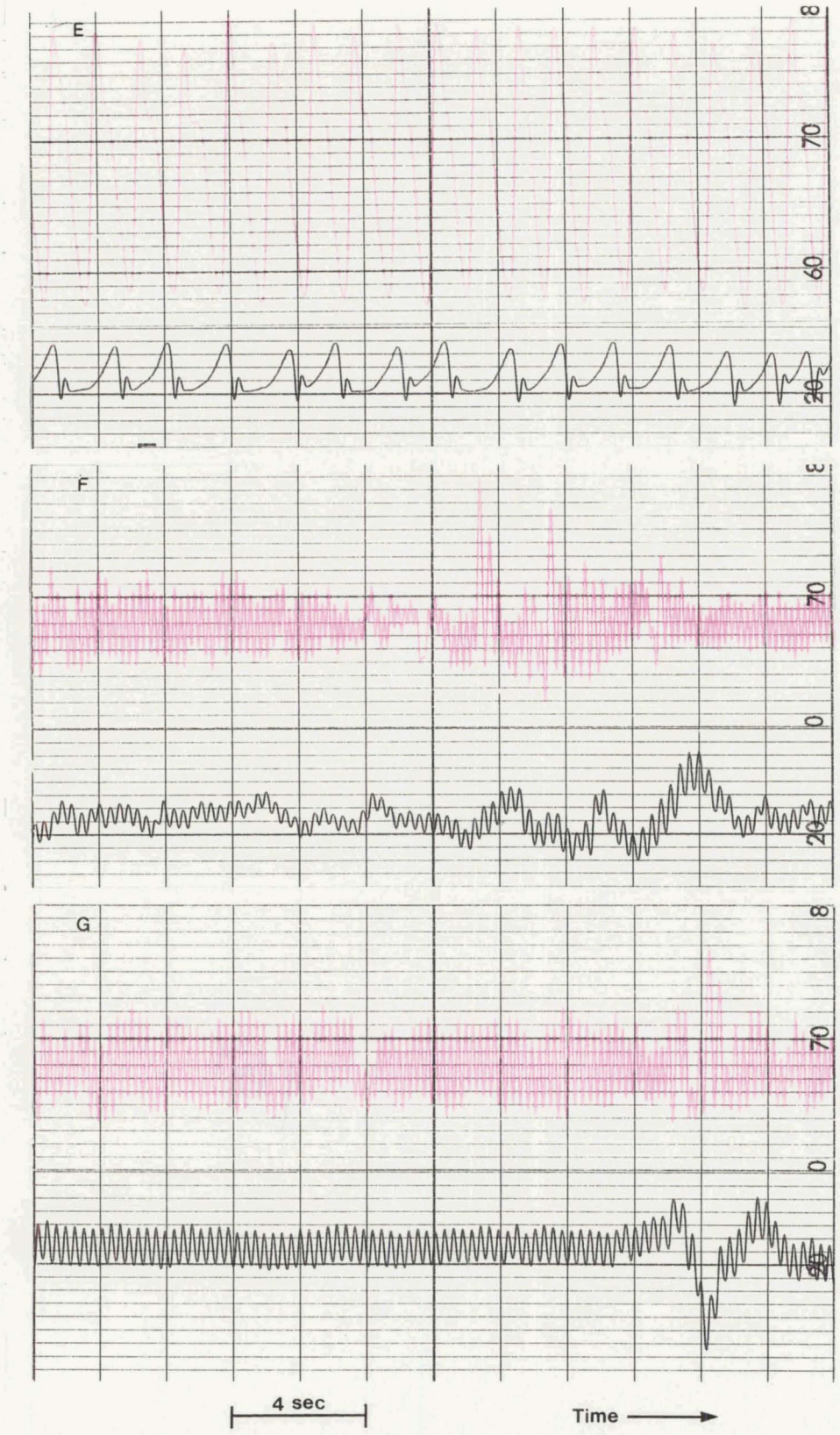

Figure 5.4 Effects of reduced oxygen tension on heart rates and ventilation frequency of ovigerous *H. rotundifrons*. There is a trend of decreased heart rate associated with the reduced oxygen tension (black line). An increase in ventilation rates is found at the beginning of exposure, then the ventilation rates decreased in associated with the reduced oxygen tension (red line). After recovery period, the heart rates and ventilation rates become increased to the normal rate. A, B, C, D and E represent the heart rates and ventilation rates in seawater with oxygen tension 150, 100, 50, 30 and 15 mmHg respectively. F and G represent the heart rates and ventilation rates after 10 and 30 minutes recovery periods in aerated seawater.

Figure 5.5 illustrates the pattern of water pressure variations recorded from left branchial cavity of ovigerous crab during progressive hypoxia. Similar to the scaphognathite beating, mean negative pressure of non-ovigerous and ovigerous crabs did not decrease until Po_2 reduced to 50 mmHg. At Po_2 15 mmHg mean negative pressure of non-ovigerous and ovigerous crabs was -4.5±1.5 and -3.5±1.5 mmH_2O compared with -5.7±2.7 and -5.2±1.0 mmH_2O of those in normoxic water respectively. During period of recovery, both forward and reversed branchial pressure were higher than those in normoxic water in both non-ovigerous and ovigerous crabs.

Both non-ovigerous and ovigerous crabs ventilated predominantly in the forward mode during in progressive and subsequent return to normoxia. However, time spent reversed ventilation seemed to decrease during the period of hypoxia and became increased during recovery period (Table 5.7).

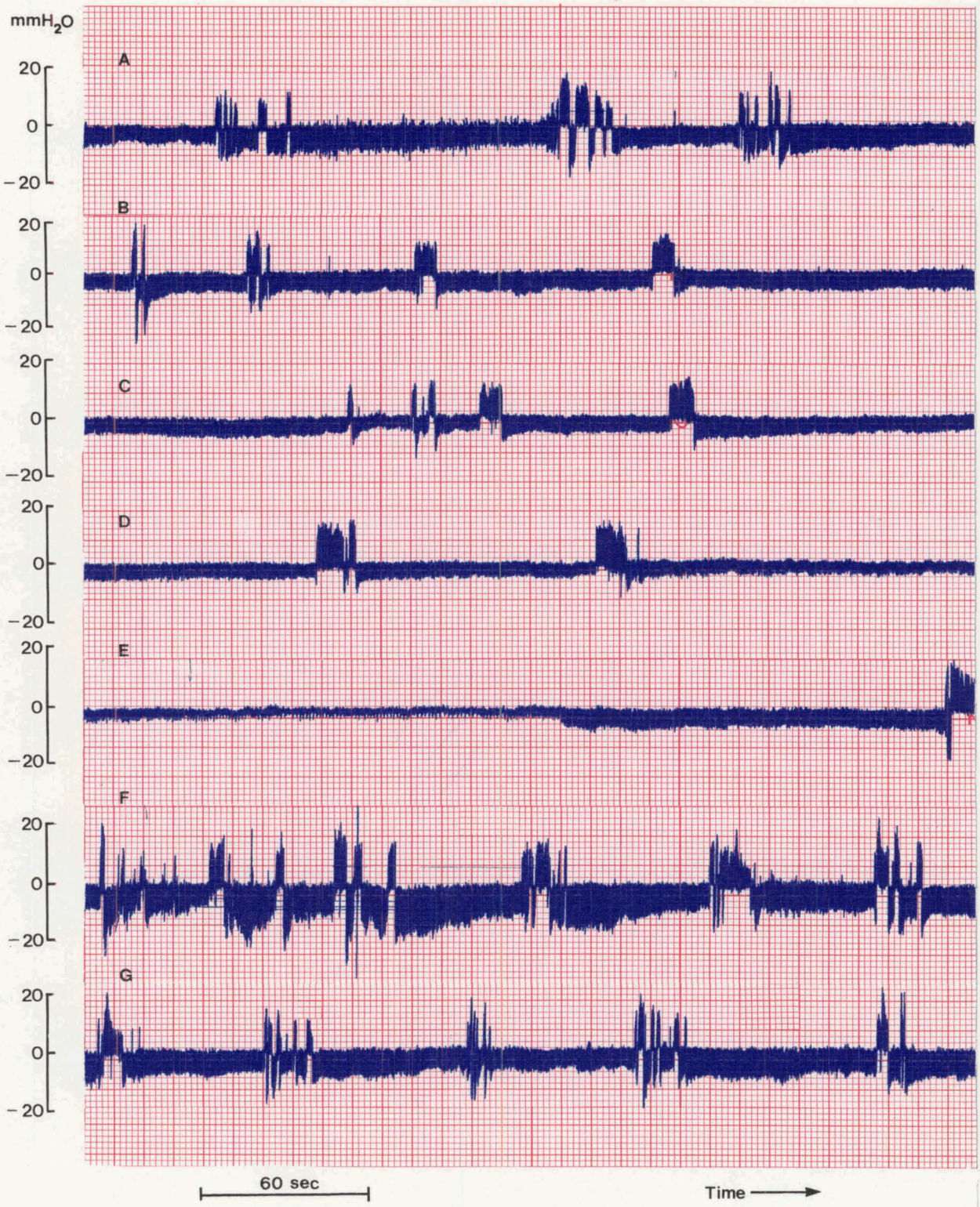

Figure 5.5 Pressure changes recorded from the left branchial chamber of an ovigerous *H. rotundifrons* during exposure to reduced oxygen tension. A, B, C, D and E represent the pressure measured in seawater with oxygen tension 150, 100, 50, 30 and 15 mmHg respectively. F and G represent the pressure measured after 10 and 30 minutes recovery periods in aerated seawater. Pressure fluctuations below zero indicate forward ventilation and above zero, reverse ventilation.

DISCUSSION

The patterns of variation in the rates of oxygen consumption in relation to ambient oxygen tension have commonly led to animals being classified either as oxygen conformers or regulators according to whether their $\dot{M}o_2$ varies in direct proportion to variations in ambient oxygen tension or whether $\dot{M}o_2$ is more or less independent over a range of ambient oxygen tension levels below air saturation (Hill, 1976). In this study, developing eggs of both species of intertidal crabs also showed the patterns of variation in the rates of oxygen consumption in relation to ambient oxygen tension. It was found that $\dot{M}o_2$ of developing eggs at the stage 5D of *H. rotundifrons* decreased with decreased oxygen tension of seawater, whereas $\dot{M}o_2$ of eggs at stage 5D of *C. lavauxi* was fairly constant down to a certain level of hypoxia after which any further decrease in oxygen tension causes a rapid decrease in $\dot{M}o_2$. Thus, developing eggs of *H. rotundifrons* and *C. lavauxi* exhibit characteristics of oxygen conformer and regulator respectively.

The rate of oxygen consumption of the regulators is divisible into an independent phase down to a critical oxygen tension below which $\dot{M}o_2$ becomes dependent upon further reduction in ambient oxygen tension. Under conditions of declining oxygen tensions in this study, developing eggs at stage 5D of *C. lavauxi* were able to maintain their rate of respiration more or less independent of the ambient oxygen tension down to a P_{crit} of about 45 mmHg. In *H. rotundifrons*, eggs showed oxyconformer which by its definition, are eggs whose $\dot{M}o_2$ varies directly with tension over the entire range from 0 mmHg to 150 mmHg (approximately 135 mmHg in this study). Thus, eggs of *H. rotundifrons* did display a zone of regulation, but their critical tension was above 135 mmHg. In a shore crab *Carcinus maenas*, Wheatly, 1981 reported that P_{crit} values of developing eggs of this crab at 11.5°C and 20°C were 50 and 80 mmHg respectively. In adult crabs, it has been reported that the relative degree of independence (i.e. the level of P_{crit}) may vary with environmental conditions and the physiological state of individual species or individuals such as the activity levels, state of disturbance or during the reproductive stage (Spoek, 1974 and Taylor, 1976). In developing eggs of crabs, the relative degree of independence may also vary in individual species and probably vary with developmental stages of eggs. Seymour and Roberts (1995) reported that the rate of oxygen consumption of frog eggs was correlated with the average Po_2 in the respiratory chambers and the rate of embryos in late development appeared to be dependent on Po_2 at all values below normoxic level than the rate of embryos in the early stages.

The significance of the critical oxygen tension at which respiratory independence is lost is not fully understood in adult crabs, but in adult fish it is thought to be due to limitations in the gas exchange system (Fry, 1957; Hughes & Shelton, 1962; Hughes, 1964). In developing eggs, the mechanism of regulation of their $\dot{M}o_2$ is even less clear. The critical oxygen tension has been reported to be dependent upon size in some species. (Herreid, 1980). In bivalves, *Laevicardium crassum* and *Mytilus edulis, M. perna* and *Arctica islandica*, large animals are more independent of P_IO_2 than small (Bayne, 1971; Taylor & Brand, 1975; Bayne, 1967). In other species, the P_{crit} value depends on the surface area for gas exchange and that the very small organisms with a relatively high surface area have no problems with O_2 conductance until very low P_IO_2 values are reached (Herreid, 1980). Dimock (1977) reported that sea cucumbers respond to hypoxia by increasing their surface area for gas exchange. Rogers (1962) calculated that nematodes may have low P_{crit} values because their elongate body and low aerobic demands permits diffusion to meet the O_2 needs in small species. In this study, the total surface area and diffusion distance of the about 20 mg of eggs used in these experiment was about three times less in *H. rotundifrons* than in *C. lavauxi*. On the other hand, normoxic mass-specific oxygen consumption rates were similar. Thus, the difference in pattern of oxygen consumption between developing eggs of *H. rotundifrons* and *C. lavauxi* may be due to differences in the surface area for gas exchange and the thickness of the diffusion barrier of eggs. As these were late stage eggs exhibiting movements of the heart and other organs it is possible that "regulation" of oxygen uptake is associated with convection changes, an aspect which requiring further study.

It is accepted that the P_{crit} relates to the point at which the respiratory gas exchange system fails to continue to provide sufficient oxygen to sustain a given activity level (Hughes, 1964), or alternatively to maintain the normoxic level of aerobic metabolism. At this stage animals may maintain their activities by shifting to anaerobiosis or they may undergo metabolic depression until oxygen is again available. The results of the lactic acid experiment indicated that developing eggs at the last stage of *H. rotundifrons* responded to hypoxic stress by lactate accumulation. This conclusion is based on the rates of egg lactate accumulation measured during hypoxic exposure. The egg lactate contents measured in this study may not accurately represent the total lactate content produced by eggs, since some may have been lost to the medium. However, it was found that the lactate concentration in eggs increased from about 0.602 $mmol.L^{-1}$ in normoxic eggs to about 10.20 $mmol.L^{-1}$ after a three hour period of hypoxic exposure. Lactate accumulation found in developing eggs of *H. rotundifrons* during hypoxic exposure suggested that eggs may also have an anaerobic metabolism.

The rates of energy production of the measured rates of O_2 uptake and lactate production in developing eggs at the last stage were compared using literature values for ATP production and energy content (Dejours, 1981). The following calculations assume that glucose is the primary substrate but the relationship would be approximately similar for other substrates.

Energy production in terms of ATP production $\equiv$ 30.5 $kJ.mol^{-1}$

Aerobic metabolism	Anaerobic metabolism
$\dot{M}o_2$ = 9.12 $\mu mol.g^{-1}.h^{-1}$	$\dot{M}_{lactate}$ = 3.057 $\mu mol.g^{-1}.h^{-1}$
*ATP/mole O_2 = 6	*ATP/mole lactate = 1
ATP energy = 1.67 $J.g^{-1}.h^{-1}$	ATP energy = 0.093 $J.g^{-1}.h^{-1}$

* ATP/ mole O_2 or lactate is derived from energy conversion :

1 mole glucose $\equiv$ 6 mole O_2 $\equiv$ 36 ATP ; 1 mole glucose $\equiv$ 2 mole lactate $\equiv$ 2 ATP

From the above Table, it can be seen that the useful energy (ATP) obtained from lactate is about 18 times less than those from O_2 uptake. In term of total energy production ($\Delta G^{o'}$), the difference is about 14 times. The details of the calculation are as follow:

Total energy production for aerobic metabolism = $9.12 \times 10^{-6} \times 2871 \times 1/6$
= 4.364 $J.g^{-1}.h^{-1}$

Total energy production for anaerobic metabolism = $3.057 \times 10^{-6} \times 198 \times 1/2$
= 0.302 $J.g^{-1}.h^{-1}$

The above calculation assumes the standard free energy change of oxidation and fermentation of glucose to be 2871 and 198 $kJ.mol^{-1}$ respectively. Assuming 1 mol O_2 $\equiv$ 1/6 mol glucose and 1 mol lactate $\equiv$ 1/2 mol glucose.

In term of substrate utilization (again assuming glucose is the main substrate), it was found that aerobic and anaerobic metabolism correspond to virtually identical rates of glucose metabolism (i.e. glycolysis):

$\dot{M}o_2$ = 9.12 $\mu mol.g^{-1}.h^{-1}$ $\equiv$ $\dot{M}_{glucose}$ = 9.12 × 1/6 = 1.52 $\mu mol.g^{-1}.h^{-1}$

$\dot{M}_{lactate}$ = 3.057 $\mu mol.g^{-1}.h^{-1}$ $\equiv$ $\dot{M}_{glucose}$ = 3.057 × 1/2 = 1.53 $\mu mol.g^{-1}.h^{-1}$

Since energy obtained from lactate is less than energy obtained from O_2 uptake. It is clear that during hypoxia, the eggs are not sustaining metabolism by anaerobiosis, but are allowing metabolic depression. Presumably, much of the energy deficit can be recovered on return to normoxia by remetabolising the lactate. However, regular exposure to hypoxia might be expected to delay development.

In vivo, the eggs are attached to female. Perhaps during the brooding period ovigerous crabs may have some special behaviours which help to maintain the level of O_2 for their eggs during the period of hypoxia. Behaviour of ovigerous crabs in response to low oxygen tension has been reported in the shore crabs *Carcinus maenas* (Wheatly, 1981). Wheatly (1981) found that in hypoxia seawater, ovigerous crabs did not show the typical emersion response as described by Taylor & Butler (1973), Taylor *et al.* (1973) and Wheatly & Taylor (1979). The stream of bubbles did not emanate from the Milne-Edwards openings which are the large openings at the base of the chelae. Instead they reversed the direction of ventilation and direct a stream of bubbles out of the openings at the base of the posteriormost pair of walking legs and thus over the developing egg mass. In this study, the behavioural responses to reducing oxygen tension was observed in *H. rotundifrons.* It was found that there was no difference in ventilation behaviour between non-ovigerous and ovigerous crabs. Upon exposure to hypoxia, both non-ovigerous and ovigerous crabs showed the behaviour which was similar to the typical emersion response by agitating and frequently stood with legs extended and ventral surface of the abdomen elevated off the floor of the chamber (Taylor & Butler, 1973; Taylor *et al.,* 1973 and Wheatly & Taylor, 1979). However, from my observations, I could not seen any stream of bubbles emanated from any parts of the body of crab. This is probably because the seawater in the experimental jar was too high and the crab could not reach the surface of the water. Thus, if the crab was observed in the shallow seawater, it is possible that they might show similar behaviour to those of ovigerous *C. maenus* reported by Wheatly (1981). The similar behaviour to *H. rotundifrons* was also reported in blue crab *Callinectes sapidus* by DeFur *et al.* (1990) that some crabs attempted to raise the anterior portion of its body above the surface of water or climb out of the water. Wheatly & Taylor (1979) suggested that such a behaviour confer a positive respiratory advantage on the crabs. By reversal of the usual direction of ventilation, the animal is enabled to utilize atmospheric oxygen. In this study, however, elevated activity ceased within a few minutes, and the animals became quiescent for the duration of the hypoxic exposure. The quiescent behaviour seemed to be an advantage for adult crabs, since their metabolic rates was reduced and thus they did not die. The developing eggs, however, would gain no direct advantage from this behaviour.

From the lactate experiment, it is apparent that developing eggs do not sustain energy production by anaerobic metabolism during hypoxia. Ovigerous crabs themselves also do not have any special ventilation mechanism to help their eggs to maintain the level of O_2. Ovigerous crabs normally buried themselves in moist sand during low tide. Such sand might be quite hypoxic due to the respiration of microorganisms. O_2 would not diffuse into interstitial water more than a few milimetres unless it was flowing or agitated. Thus, it seems that these crabs and their eggs are likely to encounter hypoxic water during the low tide period. In my experiments, however, it was found that developing eggs had abilities to survive at least three hours in extreme hypoxic water. In the field, the maximum time for each low tide period is about six hours and actually these crabs experience low tide for not more than three hours since they inhabitant the lower shore. It is thus possible that developing eggs had the ability to tolerate a few hours of hypoxic conditions during the low tide period.

The study on heart rates and scaphognathite activities found that there was no difference between heart rates and scaphognathite activities between non-ovigerous and ovigerous crabs *H. rotundifrons*. This is correlated to the results from oxygen consumption experiments (chapter 4) that ovigerous crabs did not increase their metabolic rates. Their higher metabolic rates resulted from the metabolic rates of developing eggs. Under hypoxic conditions, the heart rate and ventilation rate of both non-ovigerous and ovigerous crabs became decreased and sometimes ventilation ceased at a very low oxygen tension. In normoxic water, however, both ventilation and heart beat were virtually continuous and were not interrupted by any prolonged pauses. Wheatly and Taylor (1981) reported that generally, the response of water-breathing animals to environmental hypoxia is to increase branchial ventilation. The ventilatory requirement for water is often inversely proportional to the level of water oxygenation (Dejours, *et al.*, 1970). Taylor, *et al.* (1977) reported that crayfish exhibited the capability of increasing rate of ventilation by as much as 2.5-3.0 times the resting level during hypoxia. Butler *et al.* (1978) recorded an increase of 1.2 times in lobsters and McMahon *et al.* (1974) recorded an increase of 5 times in the crayfish *Oronectes*. McMahon *et al.* (1974) recorded a similar response of *Orconectes virilis* to long term exposure to moderate hypoxia where respiratory frequency and amplitude increased respectively by 1.5 and 3 times the normoxic levels. In this study, however, it was found that at the beginning of hypoxia the ventilation increased in both non-ovigerous and ovigerous crabs and then decreased after the Po_2 was reduced down to about 50 mmHg. Taylor (1976) reported that when *Carcinus maenas* was exposed to progressive hypoxia, oxygen supply can be maintained down to 60 mmHg below which the scaphognathite rate decreases rapidly.

Although some variability from animal to animal in mean branchial pressure was found, the pattern of water pressure recorded from left branchial cavity revealed that both non-ovigerous and ovigerous crabs ventilated predominantly in the forward mode during progressive hypoxia and subsequent return to normoxia. McDonald, *et al.* (1978) suggested that the variability in mean water pressure may have been due to differences in positions of cannulae within the epibranchial cavity. Under progressive hypoxia, the mean pressure water was found to be decreased and it was markedly increased after return to normoxia. Similarly, the time spent in reversed ventilation seemed to be decreased during the period of hypoxia and became increased during the recovery period. McMahon *et al.* (1974) found that during long term exposure to moderate hypoxia, *Orconectes virilis* showed 1.3 fold increase in the level of hydrostatic pressure in the branchial chambers.

Following the replacement of air-saturated seawater to crabs which had been exposed to very low oxygen concentrations for a period, the heart rate increased rapidly to maximal levels, returning to normal only gradually over a period of about 30 min. These results possibly indicated that there was a period of recovery from oxygen debt following such exposure. Teal & Carey (1967) have shown that in the marsh crab, *Uca pugnax*, during periods of exposure to oxygen tensions below the critical level, there is a build up of oxygen debt as a result of conversion of glycogen to lactate which is rapidly complete upon return to aerobic conditions.

In conclusion, the pattern of oxygen consumption of developing eggs of *H. rotundifrons* and *C. lavauxi* were found to be different. Developing eggs of *H. rotundifrons* exhibited the characteristics of an oxygen conformer, whereas those of *C. lavauxi* exhibited the characteristic of an oxygen regulator. This difference in pattern of oxygen consumption between developing eggs of *H. rotundifrons* and *C. lavauxi* may be due to the difference in surface area for gas exchange of eggs. In *H. rotundifrons*, both developing eggs and ovigerous females have no special ventilatory mechanism to regulate their use of O_2 during hypoxia. The only adaptation found in developing eggs is that they have abilities to tolerate the hypoxic conditions for a few hours during the period of low tide. The observations on heart rates and scaphognathite activities of adult crabs *H. rotundifrons* revealed that this species also showed a decrease in heart rate and ventilation rate in response to hypoxia which is similar to the behaviour found in other shore crabs. However, there is no difference in heart rates and scaphognathite activities found between non-ovigerous and ovigerous crabs and this is correlated with the results on rate of oxygen consumption in ovigerous crabs that the increased rate of oxygen consumption was due to developing eggs only.

CHAPTER SIX

OSMOTIC AND IONIC REGULATION DURING DEVELOPMENT OF CRAB EGGS

INTRODUCTION

Besides the problems of oxygen availability and temperature variation already considered, intertidal fauna may also be subjected to osmotic problems. At high tide animals are bathed by seawater of relatively constant composition. At low tide the salinity in tide pools and interstitial surface water may become rapidly higher or lower than seawater due to the influences of evaporation, precipitation and run off. Animals inhabiting intertidal areas are affected by the change in salinity directly and the effects can be related to both organismic and molecular changes of animals, at any stage of their life cycle.

Most marine crustaceans have body fluids that are isosmotic with the medium in which they live. When there is a change in the concentration of the medium, an animal may respond in one of two ways. The first is simply, to allow the osmotic concentration of its body fluids to correspond to that of the medium, thus remaining isosmotic with the medium; such an animal is called an "osmoconformer". Alternatively, an animal may maintain or regulate its osmotic concentration in spite of external concentration changes, such an animal is called an "osmoregulator". Even in osmoconformers, the ionic concentrations of body fluids are usually different from those outside. Thus, most animals have an osmotic or ionic gradient between the environment and the extracellular fluid and some physiological capacity for handling the water and ion movements resulting from those gradients. In both osmoconformers and osmoregulators, exposed to a sudden salinity decrease, transient differences in osmolarity exist between the internal and external media. In osmoconformers, the osmotic water movements associated with such differences are often handled by a short period of increased output from the excretory system (Binns 1969a,b; Shaw 1961; Zander 1978; Dehnel & Malley 1980; Norfolk & Craik 1980 and Robinson 1982). Osmoregulators may have much larger and sustained osmotic gradients between extracellular fluids and the environment, and in the acclimated state passive water and ion movements must be dealt with by sustained activity of ionic transport systems in the excretory organs, gills, gut, and integument.

In hypoosmotic conditions, osmoconforming animals rapidly swell in response to osmotic influx of water but, with time in the reduced salinity, most will at least partially recover the original volume. Osmoconformers usually swell less than predicted for simple osmometers. If osmotic equilibrium occurs only by water movements, the change in volume should follow the equation $P(V-b) = \text{constant}$, where P is the osmotic pressure of the dissolved substances in the extracellular fluids, V is the total volume and b is the constant (osmotically inactive material). In practice, the volume change may be even less due to solute movements. Osmoregulators, on the other hand, may show a complete volume recovery or may simply swell less than predicted (Oglesby, 1981). These whole animal responses are the result of water balance mechanisms which function at two levels, extracellular and intracellular. For regulation at the extracellular level, bulk movement of extracellular water by the excretory system, ion transport by various epithelia, and changes in integumental water permeability serve to reestablish water balance. In both osmoconformers and osmoregulators, osmotic influx of water into the extracellular compartments results in a dilution of the extracellular environment. This dilution places an osmotic stress on the cells. Thus, a second response occurs at the cellular level.

Most animal cells are capable of volume regulation to some extent when confronted with anisosmotic media. Indeed, even in isosmotic media, volume control mechanisms are at work in conditions of osmotic steady-state between the inside and the outside of the cells, to counterbalance the oncotic effects of intracellular impermeant anions. Although having easily distensible plasma membranes, animal cells are able to maintain a constant volume despite the presence in the intracellular fluid of anionic macromolecules too large to pass through the cell walls. According to the Gibbs-Donnan equilibrium theory, these impermeant compounds should lead to a distribution of permeant ions inducing an osmotic pressure with subsequent swelling and lysis unless some mechanism is at work to compensate for these effects. The most generally accepted theory is that the activity of the bulk water and consequently cell volume is largely dependent on ion movements in so-called "pump and leak" systems, which control the quantity of the intracellular ions Na^+, K^+ and Cl^-. In these systems coupled Na^+/K^+ transport plays a prominent part. During swelling, the cells gain Na^+ and Cl^- and lose K^+. During volume restoration, the accumulated Na^+ is extruded while K^+ is regained. The mechanochemical hypothesis first formulated by Kleinzeller (1972) postulates another mechanism which may help in volume maintenance. In this system cellular contractile or elastic elements exert constraining forces that "squeeze" the swollen cells back to control volume. A further hypothesis concerns changes in the state of water which could account, at least partly, for intracellular ion partitioning and for control of the activity of the intracellular water

("water structuration" hypothesis-Rorive and Gilles, 1979). In this system, the changes in water availability occurring in response to shifts in osmotic equilibrium could induce changes in the activity of structures that could account for at least part of the phenomena associated with volume adjustment (Rorive & Gilles, 1979).

When animal cells are exposed to hypoosmotic conditions, they gain water and swell. To counter the swelling and prevent osmotic lysis, the cells typically expel osmotic solute together with osmotically obligated water and the cell volume recovers back toward, but usually not reaching, the original volume level. Volume regulation in hypoosmotic media has been demonstrated in vitro, in many tissues and cell types from a variety of species, invertebrates as well as vertebrates (Gilles, 1980; King & Goldstein, 1983; Macknight, 1983). The reaction of isolated cells and tissues to hypoosmotic media is generally biphasic (Gilles, 1983). After a rapid swelling, a slow volume readjustment takes place during which the tissues resume a volume close to control. If, at the end of the readjustment process, the tissues are placed back in a control media they shrink to a volume much smaller than control. The volume regulatory decrease following swelling in hypoosmotic conditions is believed to be associated with a decrease in the amount of cell osmolytes and volume readjustment implicates changes in the amount of different intracellular osmotic effectors (Gilles, 1983). In the tissues and cell types studied up to now, the major osmotic effectors implicated in volume regulation are of two types: the inorganic monovalent ions Na^+, K^+ and Cl^- on the one hand and different organic solutes of low molecular weight such as amino acids on the other hand. The relative importance of these compounds depends on the species considered. Generally, in the vertebrates, the ions Na^+, K^+ and Cl^- can account for most of the change in intracellular fluid osmolality (Ussing, 1982; Macknight, 1983). On the contrary, various amino-compounds play an important volume regulatory role in different seawater invertebrate species (Gilles, 1975, 1979).

Unicellular organism, such as eggs and protozoans, in hypoosmotic media have a particular problem. The intracellular environment is separated from the external environment only by the plasmalemma; there is no equivalent to the extracellular buffering environment of multicellular animals. Unicellular animals are also minute organisms and therefore have a very high surface:volume ratio. Consequently, water and ions would be expected to exchange rapidly across their cell membranes. In principle, three mechanisms could counteract this tendency: (1) a highly impermeable external membrane or cell wall (2) a rigid outer wall withstanding osmotic turgor or (3) expulsion of osmotically entering water. The third process is particularly found among fresh water Protozoa. Protozoans gain water via their food and by osmotic exchange

across the plasmalemma. Water is eliminated by contractile vacuoles, which expel vesicles of intracellular fluid. The mechanism of elimination of water by contractile vacuoles is by no means fully understood but probably involves some kind of solute-coupled local osmosis, in cytoplasmic membrane systems, solute reabsorption in the vacuole and actomyosin-mediated expulsion of its contents to the exterior (Kitching, 1952; Marshall, 1969; Patterson, 1980). However, there is no evidence for contractile vacuoles in single-celled eggs of freshwater animals and, in eggs that have been studied, osmotic equilibration with the water appears simply to be slowed by a low external permeability to water and salts (Kalman, 1959; Potts & Rudy, 1969; Taylor, 1977; Mangor-Jensen, 1987). Taylor (1977) found that the exchange of ions between the medium and uncleaved eggs of freshwater *Lymnaea* is very slow, he suggested the possibility that eggs have a sufficiently low permeability to water and ions to allow compensatory devices to be delayed until the regulatory systems have been developed in the earliest cleaved stages. Similarly, eggs of salmon and trout are almost impermeable to water in freshwater, although they maintain a relatively high permeability to water in isotonic saline (Potts & Rudy, 1969). The change in permeability to water in these eggs is due to a change in the properties of the vitelline membrane (Krogh & Ussing, 1937).

Animal cell membranes are water permeable and generally not enclosed by a rigid membrane which could support a significant pressure. Therefore, they must always come to osmotic equilibrium with diluted external medium or they would burst. Normally, cells in diluted medium regulate their volume by losing some ions to limit swelling. An exception to this can be found in epithelial layers such as gills, integument or secretory organs which are hypoosmotic on one side and hyperosmotic on the other. In these cases, cell volume is presumably maintained by osmotic uptake at one surface balanced by osmotic loss across the other. Osmotic gradients must be maintained by net transcellular transport of solutes. Thus, there would be a steady flow of water and salt across the cell. Overall water balance would require return of extracellular fluid to the outside either via the excretory system or directly as found in *Lymnaea* eggs and *Hydra* (Marshall, 1969; Taylor, 1977).

Later stage eggs and embryos of some freshwater animals do appear to have abilities of osmotic and ionic regulation when confronted with anisosmotic media (Kalman, 1959; Hayes, *et al.* 1946; Potts & Rudy, 1969; Beadle, 1969; Taylor, 1973,1977; Shephard, 1987). Potts & Rudy (1969), using tritiated water, showed that freshly stripped eggs of salmonid fishes in isotonic saline are highly permeable to water, but after these eggs are shed into fresh water they become almost impermeable to water as a result of the

formation of a perivitelline fluid beneath the chorion (eggs in this impermeable state are described as "water hardened" which represents a few hours old eggs). The eggs of freshwater pulmonate molluscs are immersed in capsular fluid bounded by a membrane which permits a very rapid exchange of water and inorganic ions with the outside medium. In effect they are bathed by freshwater and the eggs and embryos themselves are actively engaged in osmotic and ionic regulation (Beadle, 1969; Taylor, 1973, 1977). Taylor (1977) reported that the developing embryos of *Lymnaea stagnalis* do not avoid the problems of osmotic and ionic regulation in a dilute medium and that ion uptake and water excretion systems are developed in the earliest cleavage stages. He found that the exchange of ions between the medium and uncleaved eggs is very slow, then it increases very rapidly during trochophore to veliger stages when the main volume increase and shell formation takes place.

Very little is known of osmotic and ionic regulation in eggs and embryos of marine invertebrate animals. Prosser (1973) suggested that eggs of marine invertebrates were isosmotic with the medium and relatively permeable. Generally, they increase in size as the salinity is decreased. Likewise, for decapod crustaceans, few data exist regarding osmoregulation in developing eggs (Pandian, 1970; Winnickii & Slomianko, 1970; Charmantier & Aiken, 1987). Based on volume changes during development, Pandian (1970) concluded that after being laid, the egg membrane of lobster, *Homarus gammarus* is permeable to water; followed by a period during which the egg membrane is almost impermeable to water; at a later developmental stage the egg membrane becomes permeable to water again. He also inferred that the egg membrane is selectively permeable to salts throughout development and the rate of salt intake is almost doubled in eggs approaching hatching. However, these inferences are based on changes in total water and salt content. Without information on unidirectional fluxes, such inferences concerning water and salt permeability cannot be accepted. In the crab *Rhithropanopeus harrisii*, Winnicki & Slomianko (1970) reported that there is an increase in egg volume (the estimated volume at hatching is about 240% of the initial volume) and decrease in specific gravity of egg throughout its development. They likewise concluded that throughout the embryonic growth, the egg membrane is permeable to water and selectively-permeable to salts. In addition, they suggested that as a result of lack of a perivitelline space these crab eggs have to take up water directly from the environment which differ from the embryos of other animal groups which take water they need from the perivitelline fluid (Winnicki & Bartel, 1967; Winnicki, *et al.,* 1968).

Osmotic regulation of embryos (intact eggs), hatching prelarvae and free prelarvae of *Homarus americanus* has been studied by Charmantier & Aiken (1987). They found that

osmoregulation of the embryos, which are surrounded by both inner and outer membranes, is hyperosmotic in seawater and dilute media. This was completely different from the regulation of hatchlings and prelarvae which are "hyperosmoconformers" (i.e. the osmotic pressure of the hemolymph is slightly higher than osmotic pressure of the medium within the ranges between 500-1100 mOsmol.kg^{-1}) and surrounded by only the inner membrane.

Developing eggs of *H. rotundifrons* and *C. lavauxi* are also enclosed by membranes. Transmission electron microscopy of the eggs of *H. rotundifrons* (in chapter 2), revealed that there are two membranes surrounding eggs in both the early and late stages. These two membranes are found to be separated by a space which probably represents the perivitelline space. It is possible that these egg membranes and the space between them function as barriers that limit the movements of water and ions between the intracellular fluid of egg cells and the external environment. Since embryos inside the eggs, especially at the very early stage possess no osmoregulatory organ (Bouaricha *et al.*, 1994), it is thus interesting to know whether these egg cells have abilities in osmo-ionic regulation or not.

H. rotundifrons and *C. lavauxi* are endemic crabs of New Zealand. Both species are commonly found inhabiting the intertidal area along the coastline of South Island. Under the natural condition, ovigerous females of *H. rotundifrons* incubate their eggs under the abdomen for approximately eight months. The very slow development of these eggs suggests that they may face acute ionic and osmotic problems during the changes in salinity at low tide period (evaporation, precipitation). In *C. lavauxi*, the incubation time for eggs of this crab is only about 2 months. This species, however normally spend most of its time above the seawater level along the shore during the low tide period. As the eggs are carried under the abdomen, they are presumably in contact with surface water at low tide. This water may be diluted by precipitation and run-off or concentrated by evaporation. The problems are presumably more severe and prolonged for *C. lavauxi*, whose habitat is near the top of the shore. Additionally the smaller eggs of *C. lavauxi* have a higher surface:volume ratio. It is therefore of interest to examine the relative capabilities for survival in anisosmotic media of eggs from the two species. Clearly if egg salinity tolerance were low the responsibility for avoidance of salinity extremes would be borne by the ovigerous female crab. Such information is not available for any intertidal crabs.

Thus, the aim of this chapter was first to document the range of salinity tolerance of eggs from both *H. rotundifrons* and *C. lavauxi* at different developmental stages.

Second, whether the eggs osmoregulated or osmoconformed was determined by studying the time course of changes in internal osmolality, of total volume, of solvent volume and of the concentrations of the cations Na^+, K^+, Ca^{2+} and Mg^{2+}. It is shown that at all stages the eggs of both species maintain a hyperosmotic condition in dilute seawater for long periods. Whether this was simply a result low permeability and slow equilibration, or a true dynamic steady state, was investigated further in the next chapter by measurement of fluxes of labelled water and sodium.

MATERIALS AND METHODS

Maintenance of developing eggs

Female crabs with eggs were collected from intertidal zone at Kaikoura during the spawning period. *H. rotundifrons* were mostly found spawning in April whereas this period commenced during mid-November in *C. lavauxi*. The collected crabs were transported to Department of Zoology, University of Canterbury and maintained in a tidal system tank in a controlled temperature room 15°C for osmotic and ionic regulation studies.

Preliminary studies (Chapter 2) revealed that the time for development of eggs of *H. rotundifrons* and *C. lavauxi* under the rearing condition at temperature 15°C were approximately 194 days and 56 days respectively. Thus, the experiments were done in eggs at different stages of development throughout their incubation time. The developmental stages of eggs of both *H. rotundifrons* and *C. lavauxi* were distinguished for this study according to the criteria described in Chapter 2.

Experimental media

Seawater at different salinities including double distilled water were used as test media for salinity tolerance tests and later on for osmotic and ionic regulation studies. The control medium was 100% seawater (34 ppt, 1027 mOsmol.kg^{-1}). Hypersaline medium (125%) was obtained by adding rock salt to natural seawater. Hyposaline media (75, 50 and 25%) was prepared by dilution of natural seawater with tap water. Double distilled water was used as 0% salinity medium. Osmolality of experimental media is shown in Table 6.1.

Table 6.1 Osmolality of experimental media.

Salinity (% seawater)	125	100	75	50	25	0
Osmolality (mOsmol.kg^{-1})	1356	1027	759	505	254	0

Salinity tolerance test

When measuring osmotic and ionic concentrations, it was necessary to know whether eggs had abilities to survive in a given salinity. Therefore, the rates of survival of developing eggs at each stages in the experimental salinities were determined before starting the measurements of osmotic and ionic concentrations of eggs.

Survival of crab eggs was determined after 24 hours exposure to six (125%, 100%, 75%, 50%, 25% and 0% seawater) different salinities of seawater and after 96 hours in 50% seawater at temperature 15°C. Survival rates were determined in *H. rotundifrons* eggs at stage 2 (blastula), stage 5A and stage 5D (yolk 2 lobes). For *C. lavauxi*, survival rates were determined in eggs at stage 2 (blastula), stage 4 and stage 5D (yolk 2 lobes).

Eggs were removed from pleopod of a female and separated into a group of twenty-five eggs. After each group of twenty five eggs were blotted to remove extraneous seawater, it was transferred to a petri-dish containing 10 mL of seawater. Six replicates of twenty five eggs were performed for each salinity. Counts of mortality were taken after 24 hours in all tested salinities and 96 hours in 50% seawater. The criteria for death were abnormal swelling of eggs and lack of movement of the heart. In eggs at blastula stage, however, it was difficult to determine the mortality due to the lack of heart-beat of embryos. Thus, after the first check which was done after 24 hours of exposure, eggs left were transferred back to 100% seawater and mortality was examined again after a further 24 hours. The number of eggs dead from the first and second examinations were then combined. Eggs were determined as being alive if they recovered their volume and appeared cytologically normal (cell boundaries visible, no abnormal vacuolation). The salinity tolerance of crab eggs were expressed as percentage survival.

Measurements of total volumes, solvent volume and osmolalities of crab eggs in ranges of salinities

Developing eggs of crabs are oval in shape. Total volumes (V) were obtained by direct measurement of the eggs, using the ellipsoid formula, (Valdes *et al*, 1991) according to the equation 6.1:

$$V=4/3\pi(L/2)(l/2)^2 \qquad (6.1)$$

where "*L*" and "*l*" are the greatest and smallest diameter respectively, measured using an eyepiece micrometer.

Different stages of crab eggs from both *H. rotundifrons* and *C. lavauxi* were exposed to 125%, 100%, 75%, 50%, 25% seawater and freshwater for 24 hours at 15°C. At the end of the exposure time, eggs were homogenized in a small Eppendorf tube and osmolality of the whole eggs was immediately measured.

The osmolality of diluted eggs was measured from a group of 50 eggs acclimated in the same media used before. Eggs were homogenized and diluted with 10 µL distilled water before the osmolality being measured. Osmolalities were measured using a Wescor 5100 vapour pressure osmometer which had been calibrated with standard solution of 290 and 1000 mOsmol.kg^{-1}. Solvent volumes (for *H. rotundifrons* only) were calculated from the osmolalities of 50 homogenized eggs diluted with 10 µL distilled water according to the equation 6.2:

$$V=\frac{wd}{50\cdot(p-d)} \qquad (6.2)$$

where, V is the solvent volume of one egg, w is the volume of water added for dilution, d is the osmolality of diluted egg and p is the osmolality of whole eggs.

Time course : Osmolality and volume changes

From the preliminary experiments, it was found that eggs survived exposure to 50% sea water for up to 96 hours. Thus, the experiments were done using 50% seawater as a medium test. Osmotic pressure of eggs was measured after 1, 3, 6, 12, 24, 48, 72 and 96 hours. The method used for measuring the osmolalities and solvent volume of eggs was as described in the previous experiments.

Measurement of cation (Na^+, K^+, Mg^{2+} and Ca^{2+}) in developing eggs

Cations were determined on different stages of developing eggs of *H. rotundifrons* and *C. lavauxi* exposed to 125%, 100%, 75%, 50%, 25% sea water and fresh water at 15°C

for 24 hours. After 24 hours of exposure time, eggs were blotted dry with filter paper and ashed overnight in a muffle furnace at 600°C on platinum foil. The ash was then dissolved in 1 molar HNO_3 and diluted with 10 mL distilled water. Determination of cation concentrations (Na^+, K^+, Mg^{2+} and Ca^{2+}) were made by a Varian Techtron 1200 Atomic Absorption Spectrophotometer using an air-acetylene flame for atomization. Samples and calibration standards contained suitable ionisation suppressants and releasing agents at the same concentrations, as indicated below. All cations were measured in the absorption mode at wavelength 589, 766.5, 285.2 and 422.7 nm for Na^+, K^+, Mg^{2+} and Ca^{2+} respectively. Concentration of cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) in experimental media is shown in Table 6.2.

Table 6.2 Ion concentration in experimental media

Salinity (% seawater)	Ion Concentration ($mmol.L^{-1}$)			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
125	620	11.13	12.26	65.53
100	451	10.17	10.08	52.27
75	339	7.04	7.80	39.78
50	234	5.12	5.38	25.73
25	117	1.98	2.74	11.96
0	0	0	0	0

Preparation of standard solutions

Na^+ and K^+

Standard solutions of Na^+ and K^+ for instrument calibration were prepared from 100 $mmol.L^{-1}$ stock solutions of NaCl and KCl. The standard working concentrations of both solutions were 0, 0.025, 0.05, 0.075 and 0.1 $mmol.L^{-1}$. To minimize the interferences, 51 $mmol.L^{-1}$ KCl and 7.5 $mmol.L^{-1}$ of $CsCl_2$ were added into the samples and the standard working solutions of NaCl and KCl respectively.

Ca^{2+} and Mg^{2+}

Standard solutions of Ca^{2+} and Mg^{2+} for instrument calibration were prepared from stock solutions of $CaCl_2$ and $MgNO_3$. The standard working concentrations of $CaCl_2$ were 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mmol.L^{-1} and of $MgNO_3$ were 0, 0.025, 0.05, 0.075 and 0.1 mmol.L^{-1}. To minimize the interferences, 41 mmol.L^{-1} of $LaCl_3$ were added into the samples and both standard working solutions of $CaCl_2$ and $MgNO_3$.

Data analysis

Rates of survival of crab eggs were expressed as percentage which then being transformed to angles using arcsine transformation. The significant difference of survival was tested by analysis of variance of the transformed data. Osmolalities of crab eggs were expressed as mean ± S.E. One-way analysis of variance was used to test for significance among treatments (developmental stage and salinity). Subsequent multiple comparisons of means were performed using the Scheffe pairwise comparisons method. Statistical significance was accepted at $P < 0.05$.

RESULTS

Salinity tolerance test in eggs of H. rotundifrons

Both early and late stage eggs had the abilities to survive 24 hours exposure to dilute seawater and even fresh water. Late stage eggs were slightly more tolerant and survived slightly lower concentrations than did early stage eggs. The mortalities of eggs at stage 2, 5A and 5D above 75% seawater were negligible. In 50%, 25% and 0% seawater, the percentage survival were 90%, 73% and 67% in eggs at stage 2, 93%, 77% and 65% in eggs at stage 5A and 99%, 95% and 85% in eggs at stage 5D respectively. There were significant differences ($P < 0.05$) in percentage survival for eggs at stage 2, 5A and 5D in 0% and for eggs at stage 2 and 5A in 25% and 50% seawater compared with those in 100% seawater (Figure 6.1). There were significant differences ($P < 0.05$) in percentage survival of eggs at stage 2 and 5A compared with those of eggs at stage 5D in 0%, 25% and 50%. In 50% seawater, more than 80% of eggs at stage 2, 5A and 5D had abilities to survive within 96 hours (Figure 6.2).

Salinity tolerance test in eggs of C. lavauxi

As in *H. rotundifrons*, both early and late stage eggs of developing eggs of *C. lavauxi* had abilities to survive for 24 hours when being exposed to dilute seawater. Late stage eggs were slightly more tolerant and survived slightly lower concentrations than did early stage eggs. The mortalities of eggs at stage 2, 4 and 5D above 50% seawater were negligible. In 25% and 0% seawater, the percentage survival were 84% and 47% in eggs at stage 2, 99% and 86% in eggs at stage 4 and 97% and 75% in eggs at stage 5D respectively. Compared with *H. rotundifrons*, developing eggs of *C. lavauxi* had more abilities to survive in 50% and 25% seawater. There were significant differences ($P < 0.05$) in percentage survival for eggs at stage 2, 4 and 5D in 0% seawater and for eggs at stage 2 in 25% seawater compared with those in 100% seawater (Figure 6.3). There were significant differences ($P < 0.05$) in percentage survival for eggs at stage 2 and 5D compared with those of eggs at stage 4 in 0% seawater and for eggs at stage 2 compared with those of eggs at stage 4 and 5D in 25% seawater. In 50% seawater, more than 95% of eggs at stage 2, 4 and 5D had abilities to survive within 96 hours (Figure 6.4).

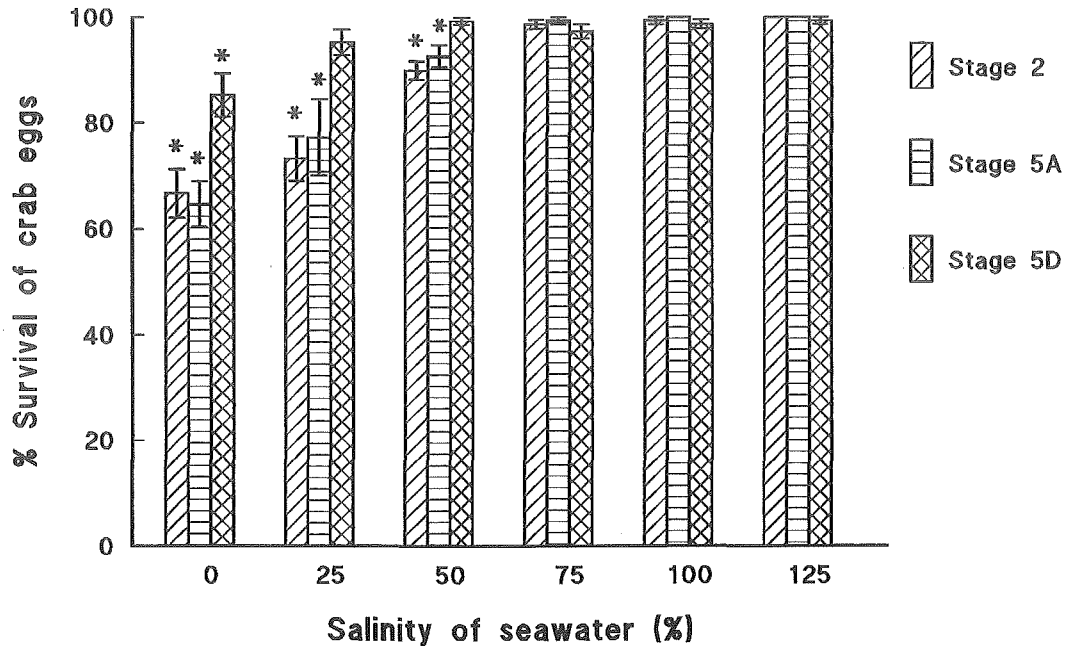

Figure 6.1 Mean (± SE) values of percentage survival of developing eggs at different stage of *Heterozius rotundifrons* after 24 hours. in different salinities. * significantly different ($P < 0.05$) from corresponding 100% seawater. (n = 150 for each bar)

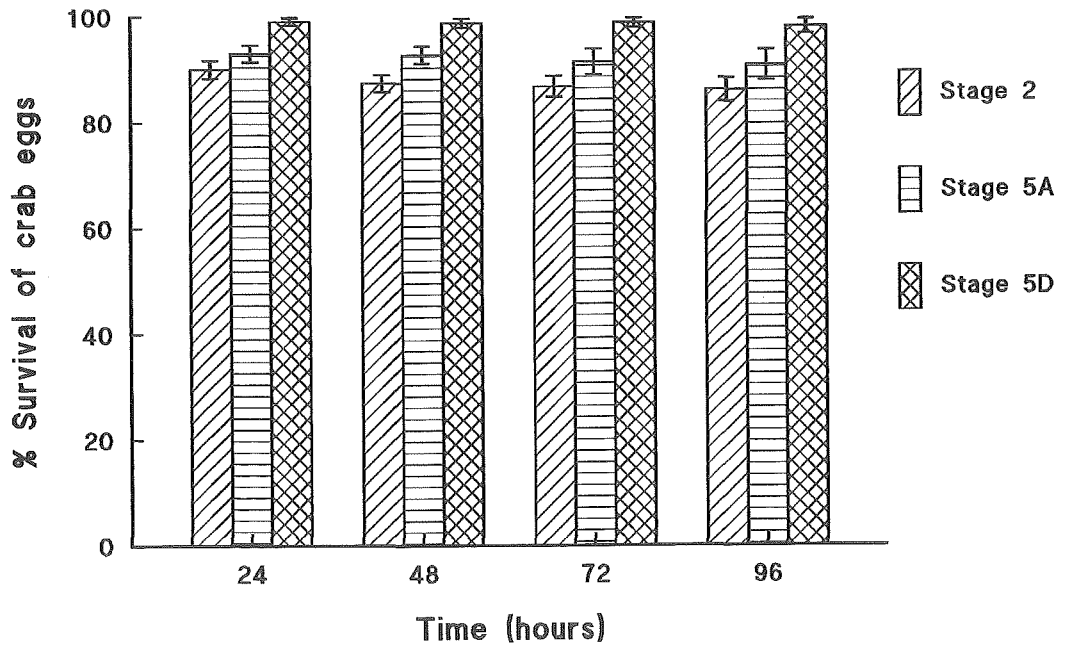

Figure 6.2 Mean (± SE) values of percentage survival of developing eggs at different stage of *Heterozius rotundifrons* after exposure to 50% seawater for times up to 96 hours. (n = 150 for each bar)

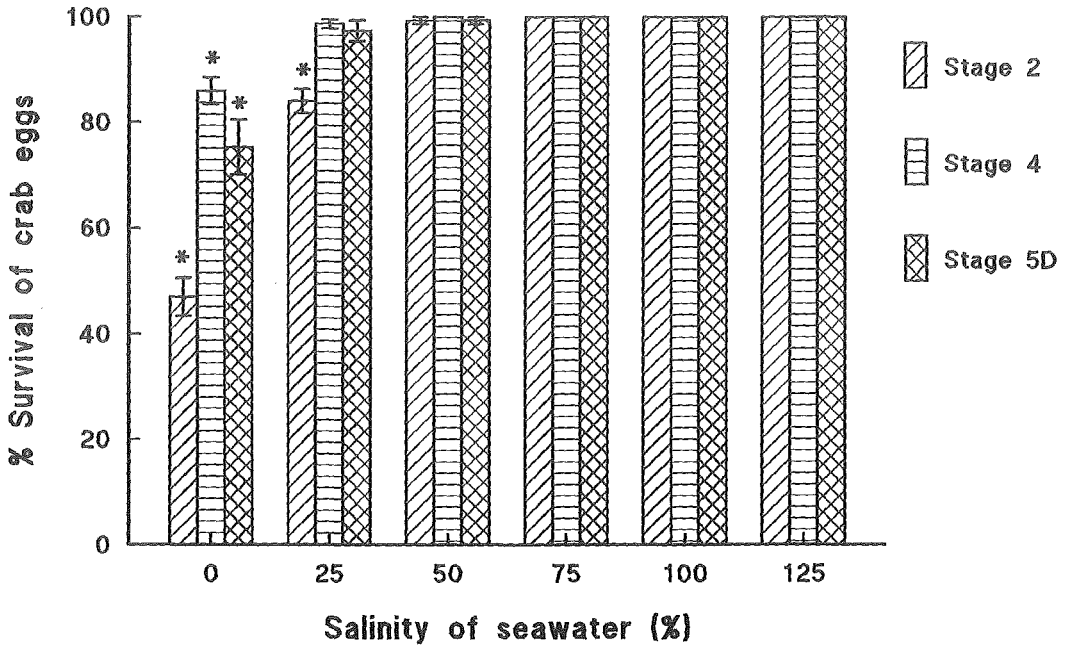

Figure 6.3 Mean (± SE) values of percentage survival of developing eggs at different stage of *Cyclograpsus lavauxi* after 24 hours in different salinities. * significantly different ($P < 0.05$) from corresponding 100% seawater. (n = 150 for each bar)

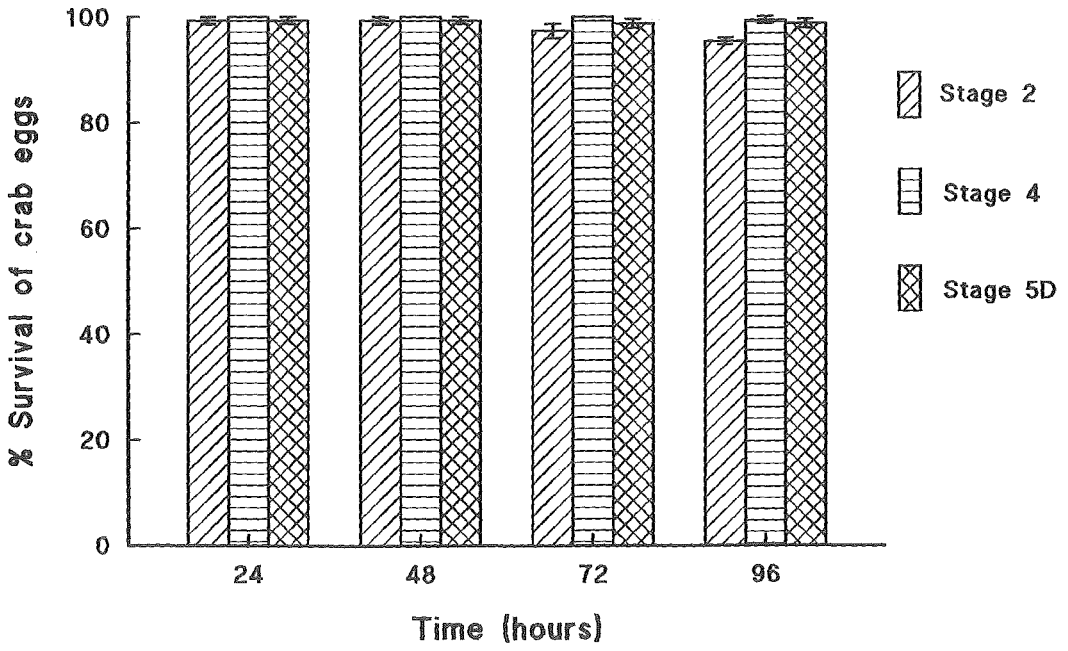

Figure 6.4 Mean (± SE) values of percentage survival of developing eggs at different stage of *Cyclograpsus lavauxi* after exposure to 50% seawater for times up to 96 hours (n = 150 for each bar)

Osmoregulation in a range of salinities for eggs of H. rotundifrons

Table 6.3 presents values for osmotic concentrations of eggs at stage 2, 5A and 5D after 24 hours exposure to 125%, 100%, 75%, 50% and 25% sea water and fresh water at 15°C. The osmolalities of all three stages of crab eggs were relatively insensitive to the external salinity and, even in fresh water, maintained osmolality equivalent to 70% sea water (Figure 6.5). Eggs at all stages were hyperosmotic to the external medium in all salinities, except that eggs at stage 5A in 125% salinity was isosmotic.

Compared with the theoretical passive line, blastulae (Stage 2) exhibited remarkably little volume change over the same range of ambient salinities (Figure 6.7). Thus, it is apparent that the egg does not behave as a perfect osmometer. Approximately half of the eggs volume in 100% seawater is osmotically inactive material (being estimated as the difference between the total volume and the solvent volume of eggs exposed to the same salinity) (Table 6.5), presumably reflecting their high yolk content. As expected, this volume (110-120 nL) remains relatively constant at an average value of 47% of the initial egg volume in 100% seawater whereas the solvent volume increased with external dilution (Table 6.5). As in stage 2, developing eggs at stage 5A showed little volume change over the same range of ambient salinities (Figure 6.8). It was found that the osmotically inactive material in eggs of this stage also remains constant (80-90 nL). When compared with eggs at stage 2, in 100% seawater, the osmotically inactive material of eggs at this stage occupied only one-third of the egg volume (Table 6.5). This is consistent with visual observation of the yolk which decreased with their development.

Total osmolytes of eggs (estimated from the product of osmolality and solvent volume) at stage 2 and 5A were 159 and 201 nOsmoles in 125% seawater and 117 and 146 nOsmoles in 0% seawater respectively. Thus, in both egg stages the quantity of osmolytes lost was only about 25% when eggs were transferred from 125% to 0% seawater.

Osmoregulation in a range of salinities for eggs of C. lavauxi

Total osmotic pressure of eggs of *C. lavauxi* after 24 hours exposure to a range of salinities is shown in Table 6.4. As in *H. rotundifrons,* the osmolalities of developing eggs of this crab are hyperosmotic relative to the medium in all salinities except in stage 5D which showed hypoosmotic to 125% seawater. (Figure. 6.6). As in *H. rotundifrons,*

Table 6.3 Osmolality of the whole eggs of *Heterozius rotundifrons* after 24 hours in different salinities of seawater and fresh water, at 15°C.

Salinity (% seawater)	Osmolality of sea water ($mOsmol.kg^{-1}$)	Osmolality of crab eggs ($mOsmol.kg^{-1}$)		
		Stage 2 (n = 4)	Stage 5A (n = 4)	Stage 5D (n = 5)
125%	1356	1403 ± 8	1332 ± 3	1404 ± 5
100%	1027	1132 ± 8	1054 ± 18	1184 ± 5
75%	759	951 ± 16	909 ± 17	1091 ± 10
50%	505	884 ± 20	821 ± 17	1011 ± 2
25%	254	817 ± 9	766 ± 12	966 ± 6
0%	0	677 ± 36	652 ± 22	713 ± 10

Table 6.4 Osmolality of the whole eggs of *Cyclograpsus lavauxi* after 24 hours in different salinities of seawater and fresh water, at 15°C.

Salinity (% seawater)	Osmolality of seawater ($mOsmol.kg^{-1}$)	Osmolality of crab eggs ($mOsmol.kg^{-1}$)		
		Stage2 (n=4)	Stage 4 (n = 4)	Stage 5D (n = 4)
125%	1356	1415 ± 17	1424 ± 11	1284 ± 6
100%	1027	1083 ± 7	1173 ± 5	1101 ± 7
75%	759	1006 ± 7	1067 ± 5	1002 ± 8
50%	505	857 ± 9	983 ± 4	936 ± 4
25%	254	766 ± 7	872 ± 5	833 ± 11
0%	0	721 ± 12	784 ± 11	694 ± 19

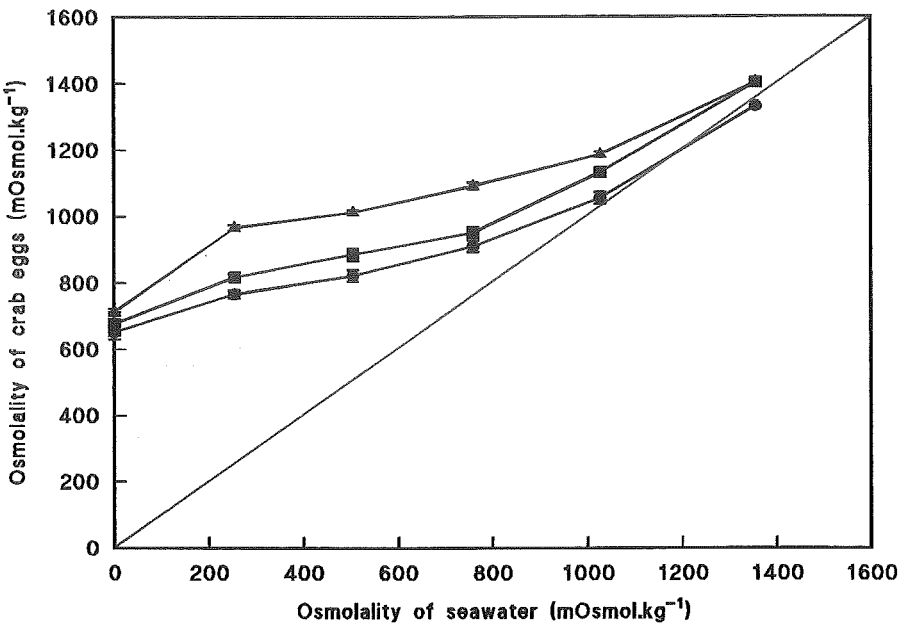

Figure 6.5 Osmolality of the whole eggs of *Heterozius rotundifrons* as a function of medium osmolality for ■ stage2 (n=4); ● stage 5A (n=4); ▲ stage 5D (n=5), after 24 h in different salinities.

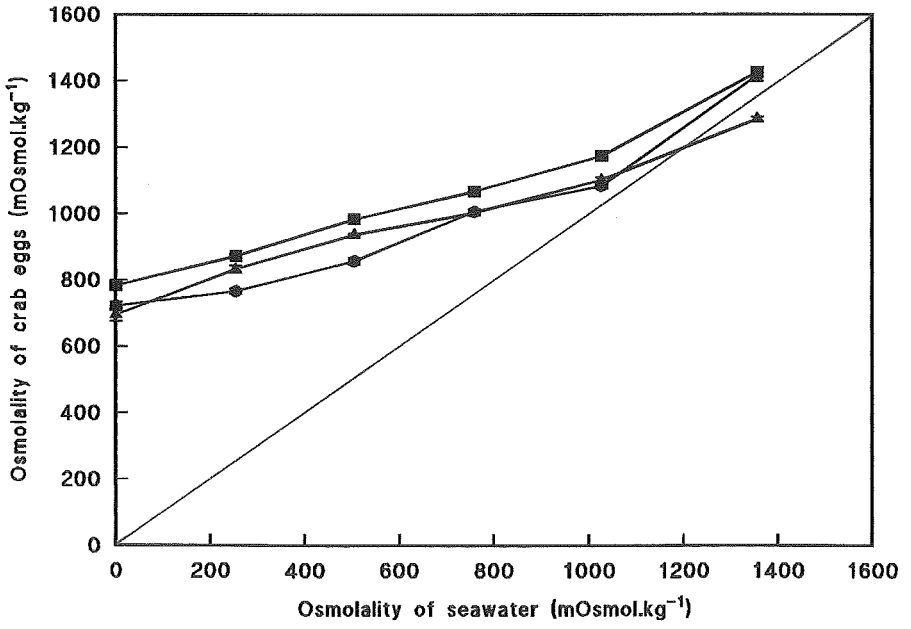

Figure 6.6 Osmolality of the whole eggs of *Cyclograpsus lavauxi* as a function of medium osmolality for ● stage2 (n=4); ■ stage 4 (n=4); ▲ stage 5D (n=5), after 24 h in different salinities.

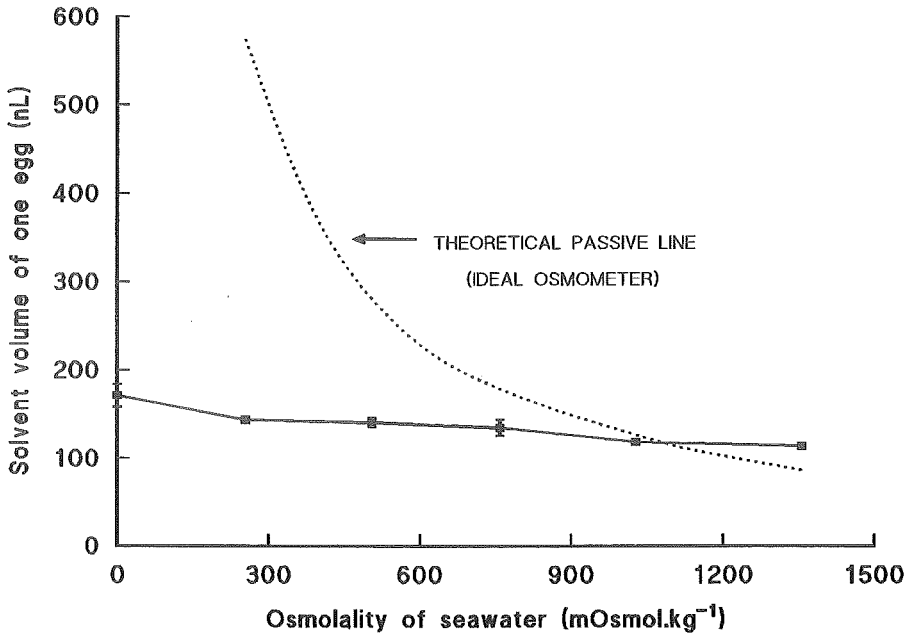

Figure 6.7 Final solvent volume of developing egg (stage 2) of *Heterozius rotundifrons* after exposure to different salinities of seawater for 24 h.

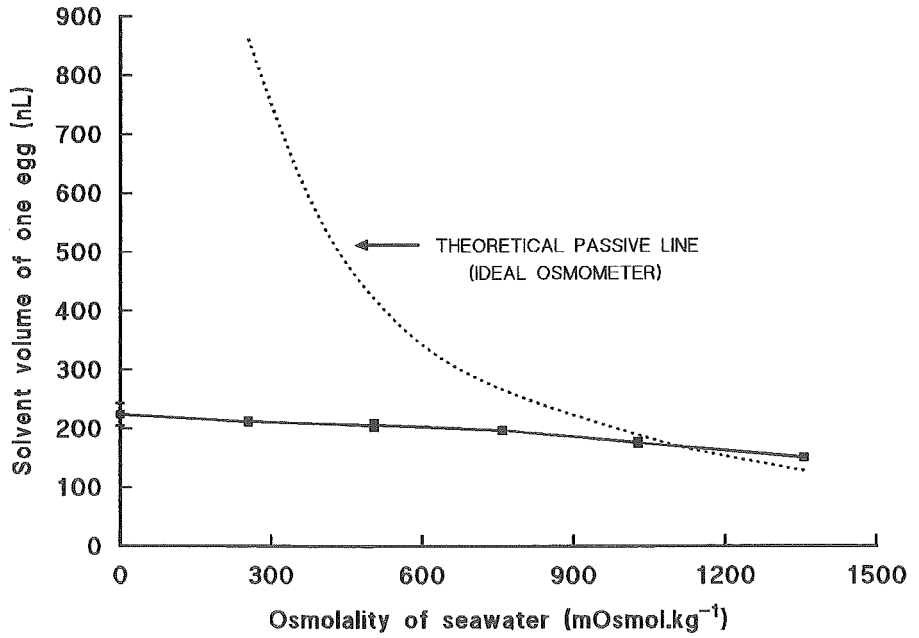

Figure 6.8 Final solvent volume of developing egg (stage 5A) of *Heterozius rotundifrons* after exposure to different salinities of seawater for 24 h.

Table 6.5 Osmolality, total volume and solvent volume of eggs at stage 2 and 5A of *Heterozius rotundifrons* after 24 hours in different salinity of seawater, at 15°C.

Stage of eggs	Salinity of seawater (%)	Osmolality of seawater ($mOsmol.kg^{-1}$)	Osmolality of crab eggs ($mOsmol.kg^{-1}$)	Total volume of one egg (nL)	Solvent volume of one egg (nL)	Osmotically inactive material (nL)	Total osmolyte (nOsmoles)	n
Stage 2	125%	1356	1403 ± 9	223 ± 5	113 ± 0	110	159 ± 0	4
	100%	1027	1132 ± 8	241 ± 5	118 ± 3	123	133 ± 4	4
	75%	759	951 ± 16	249 ± 6	134 ± 9	115	128 ± 7	4
	50%	505	884 ± 19	254 ± 7	140 ± 5	114	124 ± 4	4
	25%	254	817 ± 9	261 ± 4	143 ± 3	118	117 ± 4	4
	0%	0	677 ± 36	270 ± 3	171 ± 13	99	117 ± 5	4
Stage 5A	125%	1356	1332 ± 3	246 ± 5	151 ± 5	95	201 ± 7	4
	100%	1027	1054 ± 18	270 ± 7	176 ± 7	94	186 ± 9	4
	75%	759	909 ± 17	276 ± 8	197 ± 6	79	179 ± 3	4
	50%	505	821 ± 17	288 ± 7	206 ± 9	82	169 ± 6	4
	25%	254	766 ± 12	298 ± 9	212 ± 6	86	163 ± 6	4
	0%	0	652 ± 22	305 ± 13	224 ± 19	81	146 ± 10	4

when compared with the theoretical passive line, blastulae (Stage 2) exhibited remarkably little change in total volume over the same range of ambient salinities (Table 6.7 and Figure 6.11). Thus, in this species, the eggs also do not behave as perfect osmometers. Solvent volume and total osmolytes were not estimated in this species.

Time course of changes in osmolalities and volume of eggs of H. rotundifrons

Figure 6.9 and Table 6.6 shows changes in osmolalities, total volume and solvent volume of eggs at blastula (stage 2). It can be seen that the osmolality of eggs changed rapidly in the first few hours and thereafter declined more slowly. The total and solvent volume also changed with time. It was found that after a small increase in total and solvent volume during the first few hours, volumes plateaued (Figure 6.9). Eggs at stage 5A showed similar trends (Figure 6.10 and Table 6.6). Osmotically inactive material remained constant in the range of 110-120 nL and 80-90 nL for eggs at stage 2 and stage 5A respectively (Table 6.6).

Time course of changes in osmotalities and volume of eggs of C. lavauxi

As in *H. rotundifrons*, eggs of *C. lavauxi* survived exposure to 50% sea water for at least 96 hours and maintained both volume and osmolality (Figure 6.12). *C. lavauxi* resisted equilibration better than *H. rotundifrons*. At the end of acclimated time (96 hours), the osmolality of eggs at stage 5D was still markedly hyperosmotic (873 $mOsmol.kg^{-1}$) to the external medium (505 $mOsmol.kg^{-1}$) (Table 6.8).

Ionic regulation of eggs of H. rotundifrons

Figure 6.13 and Table 6.9 and 6.10 present the mean concentration of four cations in eggs of different stages transferred to seawater of various salinities for 24 hours. It can be seen that the overall concentrations of all four cations increased with their development and decreased as the salinities decreased. Sodium and potassium were the main cations in the eggs, sodium being consistently hypo-ionic and potassium hyper-ionic compared with the experimental seawater.

Overall sodium concentration of eggs in all five stages in 100% seawater was hypoionic compared with ambient seawater (Figure 6.13). In this salinity, sodium concentration

Table 6.6 Osmolality, total volume and solvent volume of eggs at stage 2 and 5A of *Heterozius rotundifrons* at different time in 50% seawater (osmolality = 505 mosmol.kg^{-1}), at 15 °C.

Stage of crab eggs	Time	Osmolality of crab eggs (mOsmol.kg^{-1})	Total volume of one egg (nL)	Solvent volume of one egg (nL)	Osmotically inactive material (nL)	Total osmolyte (nOsmoles)	n
Stage 2	0	1154 ± 8	246 ± 7	122 ± 5	124	141 ± 6	5
	1	1016 ± 19	252 ± 6	132 ± 8	120	134 ± 9	5
	3	971 ± 13	263 ± 8	140 ± 7	123	136 ± 8	5
	6	960 ± 12	261 ± 2	141 ± 1	120	135 ± 1	4
	12	916 ± 2	264 ± 6	143 ± 8	121	131 ± 7	5
	24	886 ± 18	273 ± 14	148 ± 7	125	131 ± 5	4
	48	820 ± 29	282 ± 23	161 ± 20	121	133 ± 12	2
	72	776 ± 14	285 ± 11	167 ± 7	118	129 ± 4	3
	96	734 ± 21	281 ± 11	170 ± 20	111	125 ± 12	3
Stage 5A	0	1068 ± 13	264 ± 8	188 ± 5	76	201 ± 4	4
	1	955 ± 18	273 ± 7	193 ± 12	80	184 ± 8	4
	3	930 ± 9	279 ± 7	191 ± 6	88	178 ± 4	4
	6	926 ± 17	284 ± 6	196 ± 9	88	182 ± 5	4
	12	867 ± 26	284 ± 6	201 ± 6	83	174 ± 5	4
	24	828 ± 24	299 ± 10	218 ± 5	81	181 ± 4	4
	48	762 ± 21	308 ± 8	223 ± 8	85	170 ± 4	4
	72	746 ± 12	313 ± 7	226 ± 6	87	169 ± 4	4
	96	708 ± 16	322 ± 4	239 ± 12	83	169 ± 6	4

Table 6.7 Total volume of one egg at stage 2 of *Cyclograpsus lavauxi* after 24 hours in different salinities of seawater, at 15°C.

Salinity of seawater (%)	Osmolality of seawater ($mOsmol.kg^{-1}$)	Total volume of one egg (nL)	n
125%	1356	8.92 ± 0.15	4
100%	1027	9.76 ± 0.00	4
75%	759	9.84 ± 0.13	4
50%	505	10.55 ± 0.27	4
25%	254	11.96 ± 0.40	4
0%	0	13.2 ± 0.37	4

Table 6.8 Osmolality and total volume of eggs, (stage 5D) of *Cyclograpsus lavauxi* at different time in 50% seawater (osmolality = 505 $mosmol.kg^{-1}$), at 15°C.

Time	Osmolality of crab eggs ($mOsmol.kg^{-1}$)	Total volume of one egg (nL)	n
0	1100 ± 7	18.71 ± 0.37	4
1	1008 ± 18	18.76 ± 0.38	4
3	1006 ± 12	19.15 ± 0.35	4
6	991 ± 14	19.13 ± 0.17	4
12	985 ± 8	19.32 ± 0.12	4
24	972 ± 6	19.47 ± 0.07	4
48	937 ± 38	19.68 ± 0.22	4
72	932 ± 10	19.82 ± 0.24	4
96	873 ± 9	19.82 ± 0.10	4

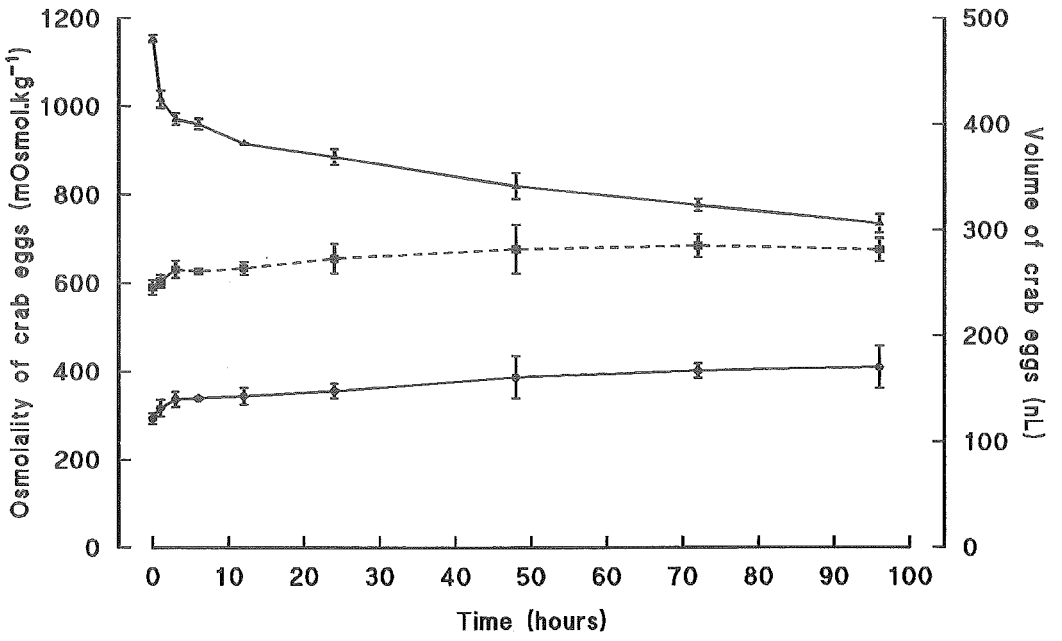

Figure 6.9 Changes in osmolalities (▲), total volume (■) and solvent volume (●) of developing egg (blastula, stage 2) of *Heterozius rontundifrons* exposed to 50% seawater (osmolality = 505 mOsmol.kg^{-1}) for up to 96 hours, at 15°C.

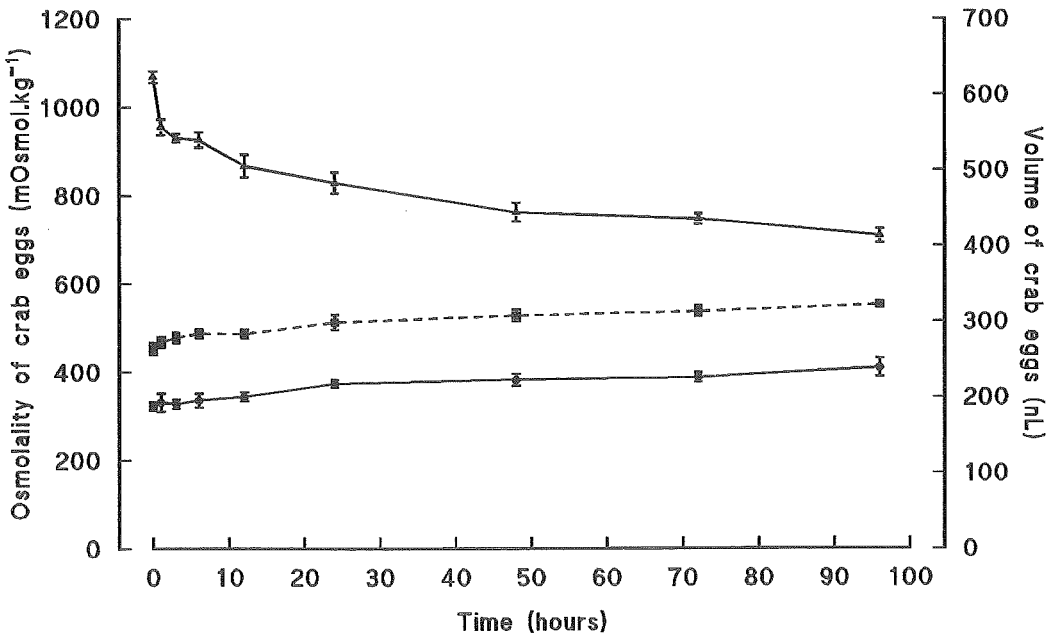

Figure 6.10 Changes in osmolalities (▲), total volume (■) and solvent volume (●) of developing egg (blastula, stage 5A) of *Heterozius rontundifrons* exposed to 50% seawater (osmolality = 505 mOsmol.kg^{-1}) for up to 96 hours, at 15°C.

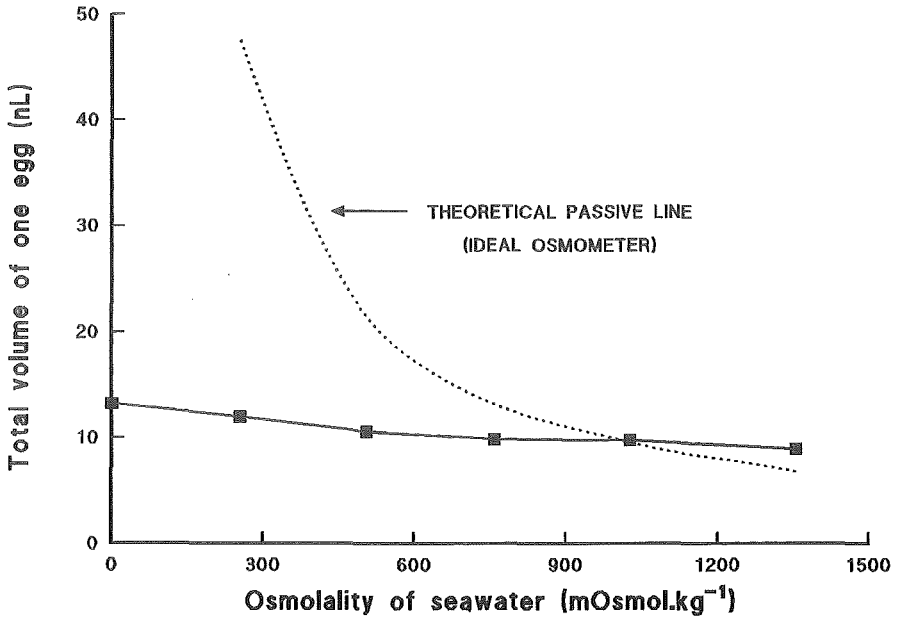

Figure 6.11 Total volume of developing egg (stage 2) of *Cyclograpsus lavauxi* after exposure to different salinities of seawater for 24 h.

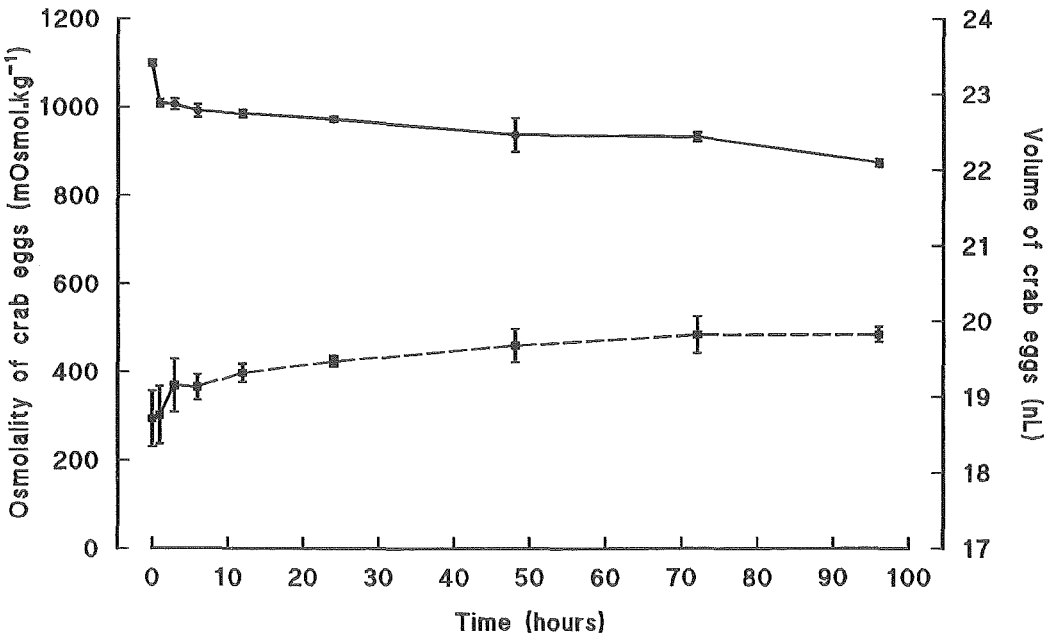

Figure 6.12 Changes in osmolalities (●) and total volume (■) of developing egg (blastula, stage 2) of *Cyclograpsus lavauxi* exposed to 50% seawater (osmolality = 505 mOsmol.kg^{-1}) for up to 96 hours, at 15°C.

Table 6.9 Concentrations of sodium and potassium in different egg stages of *Heterozius rotundifrons* after 24 hours in different salinities of seawater at 15°C.

Salinity of seawater (%)	Sodium concentration ($mmol.L^{-1}$)						Potassium concentration ($mmol.L^{-1}$)					
	Medium	Stage 2 (n=7)	Stage 4 (n=6)	Stage 5B (n= 4)	Stage 5C (n=4)	Stage 5D (n=4)	Medium	Stage 2 (n=7)	Stage 4 (n=6)	Stage 5B (n=4)	Stage 5C (n=4)	Stage 5D (n=4)
125%	620	137 ± 9	141 ± 8	139 ± 15	207 ± 4	230 ± 7	10.38	29.47 ± 1.37	48.66 ± 2.64	50.71 ± 2.71	80.11 ± 0.78	82.92 ± 3.79
100%	451	78 ± 3	81 ± 6	97 ± 8	156 ± 8	199 ± 8	9.72	28.55 ± 0.74	31.73 ± 0.83	39.69 ± 2.43	70.15 ± 3.4	80.75 ± 6.87
75%	339	25 ± 9	62 ± 8	82 ± 10	136 ± 12	168 ± 3	6.82	18.34 ± 1.18	27.76 ± 2.99	39.53 ± 5.16	66.0 ± 5.94	83.40 ± 11.81
50%	234	22 ± 2	36 ± 4	46 ± 7	119 ± 19	145 ± 4	4.66	16.51 ± 0.78	22.73 ± 2.59	24.79 ± 6.06	64.78 ± 5.83	84.79 ± 3.79
25%	117	15 ± 2	27 ± 3	36 ± 1	96 ± 15	120 ± 12	1.54	13.65 ± 0.95	19.70 ± 0.95	24.11 ± 4.01	61.10 ± 6.01	82.46 ± 11.81
0%	0	13 ± 2	15 ± 2	21 ± 4	58 ± 9	81 ± 11	0	12.29 ± 1.04	15.13 ± 2.02	15.56 ± 3.68	40.81 ± 4.97	63.70 ± 9.12

Table 6.10 Concentrations of magnesium and calcium in different egg stages of *Heterozius rotundifrons* after 24 hours in different salinities of seawater at 15°C.

Salinity of seawater (%)	Magnesium concentration ($mmol.L^{-1}$)						Calcium concentration ($mmol.L^{-1}$)					
	Medium	Stage 2 (n=7)	Stage 4 (n=6)	Stage 5B (n=4)	Stage 5C (n=4)	Stage 5D (n=4)	Medium	Stage 2 (n=7)	Stage 4 (n=6)	Stage 5B (n=4)	Stage 5C (n=4)	Stage 5D (n=4)
125%	65.53	-	19.17 ± 0.80	19.80 ± 1.40	25.41 ± 0.84	27.59 ± 1.32	12.26	-	7.48 ± 2.23	10.38 ± 1.53	14.50 ± 0.83	17.87 ± 1.14
100%	52.27	3.75 ± 0.35	13.06 ± 0.48	16.01 ± 0.72	21.56 ± 1.82	25.41 ± 1.08	10.08	3.57 ± 0.29	6.91 ± 1.50	8.06 ± 1.57	12.61 ± 0.08	16.09 ± 0.66
75%	39.78	-	8.37 ± 1.08	13.66 ± 0.58	20.59 ± 1.65	24.46 ± 1.35	7.80	-	6.15 ± 1.63	7.80 ±1.30	10.67± 2.21	15.67 ± 0.97
50%	25.73	-	5.70 ± 0.52	6.74 ± 1.17	19.14 ± 0.94	22.11 ± 1.21	5.38	-	4.92 ± 1.17	6.33 ± 1.50	10.27 ± 1.69	14.89 ± 0.88
25%	11.96	-	3.26 ± 0.34	4.87 ± 0.34	15.64 ± 1.96	18.92 ± 2.89	2.74	-	3.56 ± 0.99	4.31 ± 1.20	9.40 ± 1.29	13.31 ± 1.68
0%	0	-	2.57 ± 0.63	3.56 ± 0.80	9.15 ± 0.93	13.67 ± 2.46	0	-	4.26 ± 1.33	4.41 ± 1.31	5.98 ± 0.97	11.18 ± 1.99

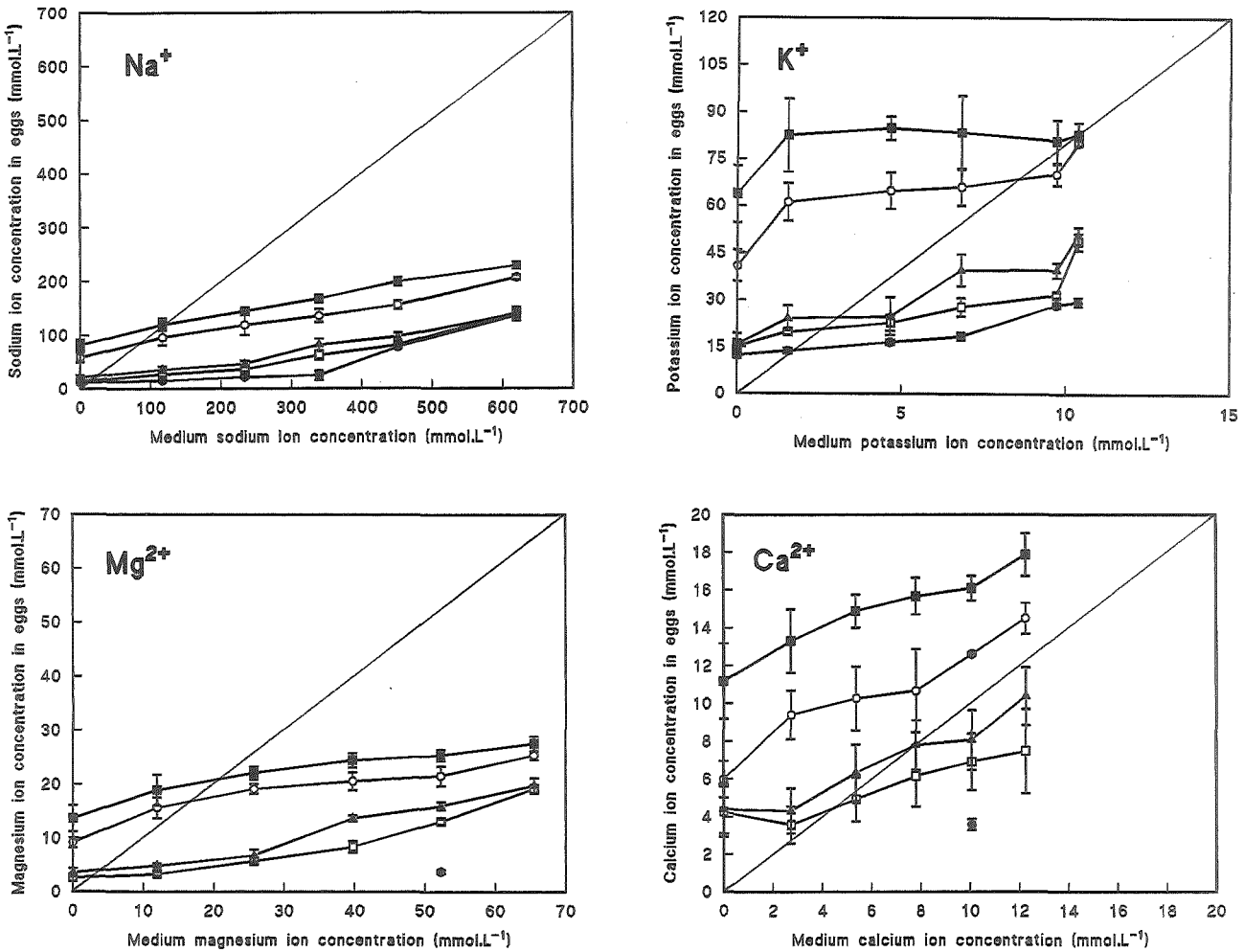

Figure 6.13 Four cation concentrations of the whole eggs of *Heterozius rotundifrons* as a function of medium ion concentration for: ● stage 2 (n=7); □ stage 4 (n=6); ▲ stage 5B (n=4); o stage 5C (n=4); ■ stage 5D (n=5), after 24 h in different salinities.

of eggs at stage 2 (blastula stage) was about one-sixth that in the medium. However, its concentration was increased before hatching to about a half of the medium. In dilute seawater, the concentration of sodium of developing eggs at both early and late stages were hypoionic to the medium, except eggs at stage 5D in 25% seawater.

Overall potassium concentration of eggs increased with development in all acclimated seawater. It was found that concentration of potassium in all five stages of eggs were hyperionic compared with ambient seawater (Figure 6.13). Eggs at the last stage showed rather constant concentrations of potassium in all salinities except in fresh water. In 100% seawater, potassium concentration of eggs at blastula stage was about three times higher than that in the medium, and increased to about eight times before hatching. In hyposalinities, potassium concentration of eggs at both early and late stages were hyperionic to the medium. .

As with sodium, the magnesium concentration of eggs in all five stages in 100% seawater was hypoionic compared with ambient seawater (Figure 6.13). In this salinity, the magnesium concentrations of eggs at the blastula stage was about one-thirteenth that in the medium. However, it was strongly increased in eggs at the last stage as its concentration was about a half that of the medium. In hyposalinities, magnesium concentrations of eggs at both early and late stages were hypoionic to the medium, except eggs at stage 5C and 5D in 25% seawater which were hyperionic to the medium.

For calcium, in 100% seawater, its concentration at the blastula stage was about one-third that in the medium, but at the end of their development, their concentration was 1.5 times higher than that in the medium. In hyposalinities, concentration of calcium in eggs at the early stages was about the same as those found in the external medium and it becomes hyperionic in the late stage eggs.

Ionic regulation of eggs of C. lavauxi

As in *H. rotundifrons*, the overall concentrations of all four cations increased with their development and decreased as the salinities decreased (Figure 6.14). Sodium and potassium were also the main cations in the eggs, sodium being consistently hypo-ionic and potassium was hyper-ionic compared with the experimental seawater (Table 6.11 and 6.12).

Table 6.11 Concentrations of sodium and potassium in different egg stages of *Cyclograpsus lavauxi* after 24 hours in different salinities of seawater at 15°C.

Salinity of seawater (%)	Sodium concentration ($mmol.L^{-1}$)				Potassium concentration ($mmol.L^{-1}$)			
	Medium	Stage 2 (n=4)	Stage 4 (n=5)	Stage 5D (n=6)	Medium	Stage 2 (n=4)	Stage 4 (n=5)	Stage 5D (n=6)
125%	620	120 ± 8	172 ± 5	199 ± 27	10.38	76 ± 17	108 ± 5	111 ± 15
100%	451	62 ± 10	135 ± 11	156 ± 29	9.72	55 ± 6	88 ± 6	95 ± 12
75%	339	43 ± 13	96 ± 15	141 ± 23	6.82	43 ± 7	83 ± 3	99 ± 16
50%	234	34 ± 4	83 ± 3	114 ± 12	4.66	38 ± 5	81 ± 6	90 ± 12
25%	117	21 ± 2	71 ± 7	102 ± 12	1.54	28 ± 4	76 ± 7	88 ± 12
0%	0	13 ± 3	37 ± 5	82 ± 10	0	35 ± 5	59 ± 6	85 ± 12

Table 6.12 Concentrations of magnesium and calcium in different egg stages of *Cyclograpsus lavauxi* after 24 hours in different salinities of seawater at 15°C.

Salinity of seawater (%)	Magnesium concentration ($mmol.L^{-1}$)				Calcium concentration ($mmol.L^{-1}$)			
	Medium	Stage 2 (n=4)	Stage 4 (n=5)	Stage 5D (n=6)	Medium	Stage 2 (n=4)	Stage 4 (n=5)	Stage 5D (n=6)
125%	65.53	25.06 ± 2.05	31.88 ± 1.88	32.16 ± 2.67	12.26	11.38 ± 5.06	18.73 ± 3.05	18.96 ± 2.04
100%	52.27	18.31 ± 3.59	26.65 ± 1.14	28.11 ± 2.11	10.08	8.04 ± 4.61	17.0 ± 2.90	15.72 ± 1.42
75%	39.78	10.94 ± 3.37	23.09 ± 5.59	26.39 ± 2.61	7.80	9.03 ± 6.33	15.77 ± 2.60	16.05 ± 2.38
50%	25.73	10.67 ± 2.57	23.53 ± 2.71	23.81 ± 1.44	5.38	8.28 ± 5.68	15.75 ± 3.11	15.59 ± 2.97
25%	11.96	5.28 ± 0.71	19.90 ± 1.97	21.05 ± 1.78	2.74	5.57 ± 3.75	16.24 ± 3.79	13.45 ± 2.11
0%	0	5.9 ± 1.66	15.37 ± 2.42	19.72 ± 1.74	0	7.91 ± 5.94	13.55 ± 2.56	13.97 ± 2.63

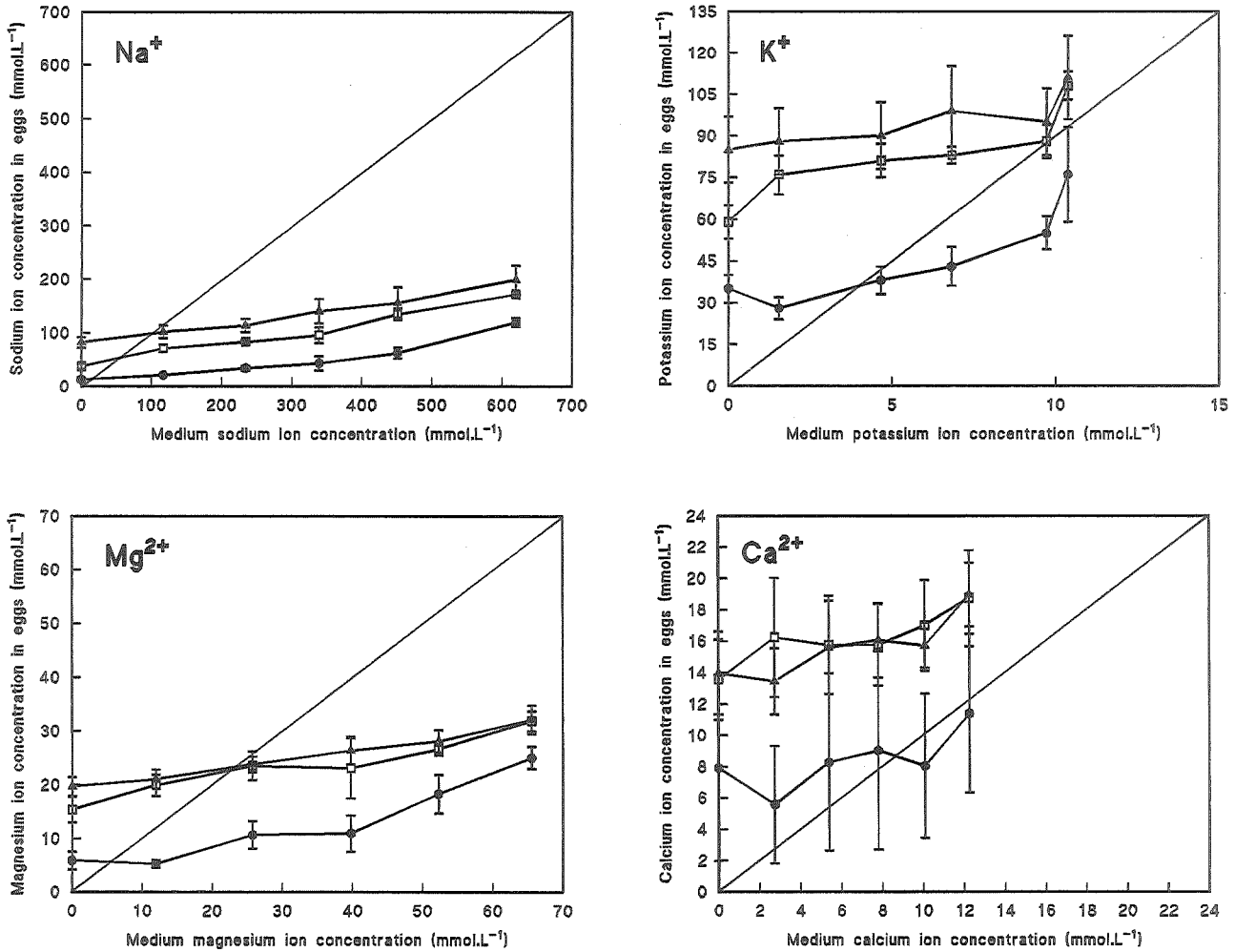

Figure 6.14 Four cation concentrations of the whole eggs of *Cyclograpsus lavauxi* as a function of medium ion concentration for: ● stage 2 (n=4); □ stage 4 (n=5) and ▲ stage 5D (n=6), after 24 h in different salinities.

Sodium concentration of eggs in all five stages in 100% seawater was hypoionic compared with ambient seawater (Figure 6.14). In this salinity, sodium concentration of egg at blastula and last stage was about one-seventh and one-third respectively of those in the medium which were similar to those found in *H. rotundifrons* (about one-sixth and a half of the medium in eggs at blastula and last stage respectively). In hyposalinities, sodium concentration of developing eggs at both early and late stages were hypoionic to the medium.

Compared with *H. rotundifrons*, both eggs at the early and late stages *of C. lavauxi* contained higher potassium in 100% seawater. The concentration of potassium of eggs at the blastula stage in 100% seawater was about six times higher than in the medium, and increased to ten times before hatching compared with those of *H. rotundifrons* which were about three and eight times higher than in the medium in eggs at the blastula and late stage respectively. As in *H. rotundifrons*, in dilute seawater, the potassium concentrations of eggs at both early and late stages of *C. lavauxi* were also hyperionic to the medium.

The magnesium concentration of eggs in of *C. lavauxi* in 100% seawater was hypoionic compared with ambient seawater (Figure 6.14). In this salinity, the magnesium concentrations of eggs at the blastula stage was about one-third of that in the medium which was higher than that found in *H. rotundifrons* (about one-thirteen of the medium), However its concentration increased to about a half that of the medium in eggs at the last stage which was similar to that of *H. rotundifrons*. As noted for *H. rotundifrons*, in diluted seawater, magnesium concentrations of eggs at both early and late stages of *C. lavauxi* were also hypoionic to the medium, except eggs at stage 4 and 5B in 25% seawater which were hyperionic to the medium.

The concentration of calcium in 100% seawater of eggs at the blastula stage of *C. lavauxi* was almost equal to that of the medium in contrast to that of *H. rotundifrons* which was only about one-third of the medium. However, at the end of their development, their concentration was about 1.5 times higher than that in the medium which was similar to that of *H. rotundifrons*. In hyposaline media, the concentration of calcium in eggs at both early and late stages was hyperionic to the medium.

DISCUSSION

Salinity-tolerance experiments indicated that eggs of *H. rotundifrons* and *C. lavauxi* wereable to survive in dilute sea water at all stages of development. When acutely exposed to reduced salinity, late stage eggs were more tolerant and survived lower concentrations than did early stage eggs. Similar relationships existed when these eggs were acclimated for 96 hours to 50% salinity. It is possible that this reflects the development of osmoregulatory organs in eggs at the later stages. Bouaricha, *et al.* (1994) studied ontogeny of osmoregulatory structures in the shrimp *P. japonicus* and reported that differentiated osmoregulatory structures are not observed in the early larval stages, but in the next larval stages, zoea and mysis, gills and epipodites are developed as buds and osmoregulatory epithelia are observed in the branchiostegites and pleurae of these larval stages.

In all salinities of seawater, eggs at all stages, except stage 5A (for *H. rotundifrons*) and stage 5D (for *C. lavauxi*) maintained their osmotic pressure higher than the medium (Table 6.3 and 6.4). Likewise, in *Homarus americanus*, it was found that hyperosmotic regulation was inferred in embryos (intact eggs) in dilute media. Hatchlings and pre-larvae were observed to be "hyperosmoconformers" (i.e. the osmotic pressure of the hemolymph is slightly higher than osmotic pressure of the medium within the ranges between 500-1100 mOsmol.kg^{-1}) (Charmantier and Aiken, 1987). Since the intact eggs of *H. americanus* are surrounded by both inner and outer membranes, Charmantier and Aiken (1987) concluded that the rupture of the outer membrane causes the hatchling and prelarval osmotic presssure to decrease. By comparison, the hatchlings and prelarvae surrounded by the inner membrane only.

The osmolalities of crab eggs decreased with the decrease of salinity of seawater, and the solvent volume increased as water moved into the egg cell by osmosis. Figure 6.7 and 6.8 show that the changes in solvent volume are extremely small compared with the theoretical line of ideal osmometer. These observations suggest that the eggs of *H. rotundifrons* did not behave as an osmometer which is similar to most living cells (Lucké & McCutcheon, 1932). Animals would act as a perfect osmometer if they were permeable to water but not to ions. Any deviation from perfect osmometry indicates a significant ion permeability and/or ionic regulation.

During acclimation of crab eggs to diluted seawater, there was a decrease in the osmotic pressure of eggs. This decrease may be accounted for by changes in the osmotically

active solutes (osmolytes) since the total osmolyte in eggs decreased with osmolality. Both inorganic ions and small organic molecules such as free amino acids are the main osmotically active solutes in organisms (Yancy *et al.*, 1982). For eggs at the early and late stages of *H. rotundifrons* in 100% seawater, the osmolality due to sodium, potassium, magnesium and calcium is approximately 214 and 525 mOsmol.kg^{-1} (using an osmotic coefficient of 0.9 and monovalent anions) respectively. The corresponding values for *C. lavauxi* are approximately 282 and 570 mOsmol.kg^{-1} respectively. Thus, the remainder of the osmotic pressure of eggs (about 75% and 50% of the total osmotic pressure in early and late stage eggs of both species repectively) must be contributed by other osmotically active material such as free amino acids, yolk products and other organic metabolites. Schoffeniels and Gilles (1970) reported that crustacean cells are generally assumed to be isosmotic with the blood and organic compounds account for about half of the total intracellular osmotic pressure. Shaw (1958) found that in muscle cell of *Carcinus*, free amino acids and other similar compounds play an important part in the osmotic activity of the cells accounting for over 60% of the total osmotic activity.

Gerard and Gilles (1972) found that amino acids play an important role in the isosmotic intracellular regulation process which occurs during acclimation of crab *Callinectes sapidus* from seawater to 50% seawater. In *H. rotundifrons,* osmolality of eggs at stage 2 decreased from 1132 mOsmol.kg^{-1} in 100% seawater to 884 and 817 mOsmol.kg^{-1} in 50% and 25% seawater respectively, whereas the estimated component of the osmolality due to sodium, potassium, magnesium and calcium decreased from 214 mOsmol.kg^{-1} in 100% seawater to about 76 and 55 mOsmol.kg^{-1} in 50% and 25% seawater respectively (using data from Table 6.3, 6.9, 6.10 and assuming an osmotic coefficient of 0.9 and monovalent anions) respectively. Clearly, the change in ion concentrations in the eggs with medium dilution can account for at most about half of the change in osmolality. A similar conclusion can be drawn for stage 5 eggs. Gerard and Gilles (1972) proposed that the intracellular osmotic pressure due to the inorganic ions in muscle tissue of crab *Callinectes* is only slightly modified in hyposalinity, thus indicating that they do not play an important part in the regulation. Since the main osmolytes in eggs are free amino acids, yolk products and other organic metabolites, it is possible that these compounds play an important role in egg volume regulation which is similar to isosmotic intracellular regulation found in crab muscle (Gerard and Gilles, 1972). Amino acid mediated volume regulation has also been reported in two molluscan tissues in response to low salinity. In these tissues cell volume regulation is accomplished by an efflux of specific amino acids from the cell (Pierce and Greenberg, 1972, 1973, 1976; Amende and Pierce, 1980). In crab eggs, it is thus possible that protein, free amino acids and some organic compounds might be lost into the medium. Alternatively low molecular

weight metabolites might be catabolyzed or incorporated into macromolecules. It is important to distinguish between these alternatives which have quite different implications for the overall metabolism and energy balance during embryonic development.

Measurements of the four cations by AAS in developing eggs at different stages after 24 hours exposure to different salinities at 15°C found that internal ions are retained and regulated differently by different stage of eggs. The overall concentrations of all four cations increased with their development and decreased as the salinities decreased. These corresponded with the decrease in total osmolytes. Sodium and potassium were found to be the main cations in the eggs. Sodium was consistently hypo-ionic and potassium hyper-ionic compared with the experimental seawater. Sodium and magnesium concentrations changed at a proportionally greater rate with dilution than did potassium and calcium. These observations are consistent with locations for most of the sodium and magnesium in a small compartment between the permeable outer egg membrane and the embryo itself and with an embryonic (in the cells, blastocoel or yolk) location for most of the potassium and calcium.

Sodium concentration in eggs of *H. rotundifrons* and *C. lavauxi* changed only slightly during development. This suggested that sodium is not the main ion in embryonic tissue. However, the increase in concentration of sodium with development suggested that sodium was taken up into the eggs during development. Sodium concentration in eggs at the early stage of *H. rotundifrons* and *C. lavauxi* in 100% seawater is about six times and seven times respectively less than in the medium and the concentration increased to about three times less than the medium in eggs at the late stage in both species.

Potassium concentration in eggs at the early stage of *H. rotundifrons* and *C. lavauxi* in 100% seawater is about three times and six times respectively higher than in the medium and the concentration increased to about eight times and ten times respectively in eggs at the late stage. The hyperionic and pronounced increase in potassium concentration in eggs with their development suggested that potassium is the main cation in the cell tissue of embryo, as cells normally have high potassium concentration. Leader and Bedford (1978) also reported the hyperionic regulation of intracellular potassium in adult *C. lavauxi*, which is 141.6 $mmol.kg^{-1}$ intracellular water, and haemolymph potassium is 9.6 $mmol.L^{-1}$ which is isoionic with the medium.

Developing eggs of both *H. rotundifrons* and *C. lavauxi* regulated magnesium hypoionically except eggs at the later stages (stage 5C and 5D for *H. rotundifrons*, stage 4 and 5D for *C. lavauxi*) in 25% sea water and eggs at all stages in fresh water (for *H. rotundifrons*). Magnesium concentration in eggs of both species increased with their development. In *C. lavauxi*, the concentration of this ion in developing eggs at the last stage is about 28.11 $mmol.L^{-1}$ compared with haemolymph magnesium in adult crabs which is about 35 $mmol.L^{-1}$. (Leader and Bedford, 1978). Hyporegulation of magnesium is the most universal feature of ionic regulation in crustacean blood (Engelhardt and Dehnel, 1973). A possible correlation exists between the locomotory activity of a particular crustacean species and its blood tonicity for this ion. Those with low values of magnesium tend to be more active and capable of faster movement than those with high blood concentrations (Lockwood, 1962; McFarland and Lee, 1963).

The increase in calcium concentration in eggs with their development may be related to a need for Ca^{2+} in integument formation during development. Calcium concentration in eggs became hypertonic in all experimental salinities in eggs at stage 5C and 5D of *H. rotundifrons* and stage 4 and 5D of *C. lavauxi*. In last stage eggs of *H. rotundifrons* and *C. lavauxi*, the concentrations of calcium were 16.09 and 15.72 $mmol.L^{-1}$ respectively. These are higher than haemolymph calcium reported in *C. lavauxi*, which is 12.1 $mmol.L^{-1}$ (Leader and Bedford, 1978).

In conclusion, the developing eggs of both *H. rotundifrons* and *C. lavauxi* have abilities to maintain a hyperosmotic condition and resist swelling in dilute seawater for a long periods. Whether such behaviour reflects simply a low permeability and slow equilibration, or a true dynamic steady state is unclear. This is investigated further in the next chapter in relation to the fluxes of labelled water and sodium.

CHAPTER SEVEN

SODIUM AND WATER EXCHANGES IN CRAB EGGS

INTRODUCTION

With respect to their osmotic environment eggs fall naturally into two classes; those which show a considerable degree of independence of the osmotic environment, represented by amphibian, avian, reptilian, teleost and fresh water invertebrates and those which are essentially in osmotic equilibrium with the surrounding medium such as eggs of marine invertebrates.

In chapter 6, it was demonstrated that the eggs of *H. rotundifrons* and *C. lavauxi* remain hyperosmotic and hyperionic to dilute seawater for several days. A hyperosmotic animal encounters two physiological problems: (1) water tends to flow into the cell because of the higher solute concentration inside; and (2) solutes tend to be lost because their inside concentration is higher and also because the water that enters must be excreted and carries some solutes with it. In principle, there are three possible mechanisms whereby animal cells could maintain a hyperosmotic internal environment: (1) development of a closed system with an impermeable barrier to the passage of water and ions across the surface; (2) development of a high internal hydrostatic pressure which would oppose the osmotic uptake of water; and (3) the performance of osmotic work involving active uptake of solutes and active excretion of water.

Yonge (1937) found that the developing egg of *Homarus vulgaris* is enclosed with membranes which consist of two layers. He considered the outer membrane to have properties identical to those of superficial cuticle of the integument (limited permeability), whereas the inner membrane was thought to be chitinous and freely permeable. Pandian (1970a) inferred changes in permeability of egg membrane to water and salt throughout development of eggs of *Homarus gammarus* based on observations of changes in salt (ash) and water content. He suggested the sudden change in egg permeability to water may be caused by either the accumulation of excretory substances and/or secretion of substances of osmotic value by special glands or gland cells in the developing embryo at the particular stage, or a special hatching enzyme whose function is the chemical alteration of the egg membrane. However, without measurements of unidirectional water or solute fluxes, inferences concerning permeabilities are premature.

There is certainly evidence that adult crustaceans can lower their integumental permeability to salts and water in exposure to lower salinity. Capen (1972) and Hannen

& Evans (1973) studied the decrease in diffusional permeability of water in crustaceans exposed to dilute salinities. They concluded that a variety of euryhaline crustaceans (i.e. *Carcinus maenus, Rhithropanopeus harrisii, Hemigrapsus nudus* and *Limulus polyphemus*) are capable of decreasing their "apparent permeability" to water upon exposure to a hypoosmotic medium. By measuring the uptake of radioactively labelled water into the hemolymph, they concluded that the reduced exchange of water could have resulted from a reduction in epithelial permeability to water. It is possible such permeability changes protect euryhaline crab eggs but as yet there is no information on this.

When animal cells such as erythrocytes are placed in a hypertonic solutions they shrink and in dilute salt solution they swell. Eventually erythrocytes become hemolyzed and lose their hemoglobin in excessively dilute salt solutions (Ponder, 1948). Plant cells, like animal cells, are also extremely permeable to water. However, they are able to exist in hypotonic salt solutions without swelling by developing a high internal hydrostatic pressure which opposes osmosis. Thus, the cells of a fresh water plant like *Elodea* are normally turgid (Dainty, 1963). The protoplast is held firmly against the cell wall. If the plant cells are placed in a concentrated salt solution they lose water and become plasmolyzed, but in fresh water each cell regains water and the protoplast again fills the space within the cell wall. The rigid cell wall, limiting the volume, prevents excessive uptake of water. However, if the cell wall is first digested by the enzyme cellulase, then the naked protoplast swells with uptake of water even to the bursting point (Ruesink and Thimann, 1966). Ostensibly, the tough outer membranes of crab eggs appear to play a similar role. In dilute seawater, cleaving embryos are closely opposed to the taut external membranes. In 125% seawater they shrink away from the outer membrane (Figure 2.10). However, direct measurements of the internal pressure of crab eggs indicate that it could not account for observed differences in osmolality (Taylor & Leelapiyanart, unpublished)

In summary, three general mechanisms could account for the observations of a hyperosmotic state in crab eggs in dilute seawater (and excess internal oncotic pressure in normal seawater). One of these mechanisms (internal turgor) is not supported by direct measurements of internal pressure in *H. rotundifrons* eggs (approximately 30-90 torr equivalent to 2-5 $mOsmol.kg^{-1}$ in 50-100% seawater; Taylor & Leelapiyanart, unpublished). Assessment of the other two mechanisms (closed system or active regulation) requires information on water and solute permeabilities. Such data are reported in this chapter based on the influx of the tracers tritiated water and ^{22}Na. Measurements of very low water and ion permeabilities (turnover times in the order of

days) would support the hypothesis that the eggs were behaving as closed systems (equilibrating extremely slowly). Turnover rates for either water or sodium in the order of hours, or less, would imply a dynamic steady state involving metabolically linked ion uptake and/or water excretion.

The kinetics of water and sodium exchanges also provide further information on their compartmentalization within the egg and the location of likely permeability barriers.

MATERIALS AND METHODS

Water influx of developing eggs of H. rotundifrons

The influx of water by developing eggs *H. rotundifrons* was measured with tritiated water (THO). Batches of eggs from a single crab were acclimated to 50% and 100% seawater for 24 hours. After estimating the mean egg volume microscopically (chapter 2), the eggs were transferred to a large volume (>100 times the volume of the eggs) of labelled 50% or 100% seawater (88 $\mu Ci.mL^{-1}$ or 3.26 $MBq.mL^{-1}$). Single eggs were removed at known time intervals (0.5, 1, 3, 5, 10, 20, 40, 60, 90, 120, 180, 300 and 1440 min respectively) in 1-2 µL of fluid and transferred directly to filter paper. As soon as external water disappeared (<1sec), the egg was transferred to a 1.5 mL Eppendorf tubes and macerated with a needle in 100 µl distilled water. One mL aqueous scintillation fluid (Beckman MP) was added to each tube before measuring the activity of tritium by liquid scintillation counting (Wallac 1410, correction for quenching applied). Two or three replicates of egg samples and three replicates of medium samples were performed at each time in each run.

Sodium influx of developing eggs of H. rotundifrons

Measurements of sodium influx in *H. rotundifrons* eggs were made using ^{22}Na as a tracer using an essentially similar procedure except that eggs were removed, blotted and counted in groups of 10 eggs. One egg sample and three replicates of the medium sample were performed at each time in each run. Experiments were done in seawater with the sodium concentration of 230 and 450 $mmol.L^{-1}$ (50% and 100% seawater respectively) labelled with ^{22}Na (3 $\mu Ci.mL^{-1}$ or 0.11 $MBqmL^{-1}$). Radioactivity was measured using a well counter and scaler (Ortec) with a narrow window set to the ^{22}Na energy peak (background 7-10 counts/min).

Data Analysis

Influx curves for THO and ^{22}Na (see Results section) revealed fast and slow components to both fluxes suggesting the eggs contained at least two pools of water and sodium, exchanging with the medium at different rates. Thus influx data were analysed in terms of a double exponential function of the form:

$$A_t = B(1 - e^{-k_B t}) + C(1 - e^{-k_C t}) \qquad (7.1)$$

where A_t is the quantity of tracer (dpm) taken up by the egg at time t (h). B, C, k_B and k_C are constants. At equilibrium, $A_t = A_\infty = B + C$.

There are several possible models for steady state exchange of two-compartment systems with a third infinite compartment, and with each other (Atkins, 1969; Shipley and Clarke, 1972; Taylor, 1977). Although influx and efflux are always described by two exponentials, the parameters B, C, k_B and k_C are equal to actual pool sizes and rate constants only under special circumstances, e.g. if the pools exchange independently with the medium (Figure 7.1, model 1). A more plausible model, consistent with the morphology (Figure 7.1, model 2, see also Discussion) envisages an outer rapidly exchanging pool of water and sodium, associated with the egg membranes. An inner slow pool associated with the embryo and/or yolk exchanges with the medium only through the first pool.

In model 2, the parameters of the influx equation 7.1 do not generally represent the actual pool sizes and rate constants. However, they will approximate these values if the influx and rate constant for the outer pool (B) are much greater than for the inner pool (C). Labelling of B is essentially complete before any significant quantity of tracer moves through to C. As shown in the Results section, this appears to be the situation in the eggs examined here. Thus after a short time, B is at equilibrium and equation 7.1 becomes:

$$A_t = B + C - C \cdot e^{-k_C t}$$

$$\ln(A_\infty - A_t) = \ln C - k_C t \qquad (7.2)$$

Values of total equilibrium tracer activity of the eggs (A_∞ dpm) were obtained after 5 hours (early egg stages) or 24 hours (later stages) exposure to the labelled medium. Values of the equilibrium tracer content of the inner pool (C, dpm) and rate constant for exchange (k_C, h^{-1}) were obtained by linear regression of influx data for the first hour (THO) and the first three hours (^{22}Na) according to equation 7.2. The outer pool activity, $B = A_\infty - C$, dpm. Equilibrium activities were converted to exchangeable water volume (EWV, nl) or sodium contents (Na, nmol) as follows:

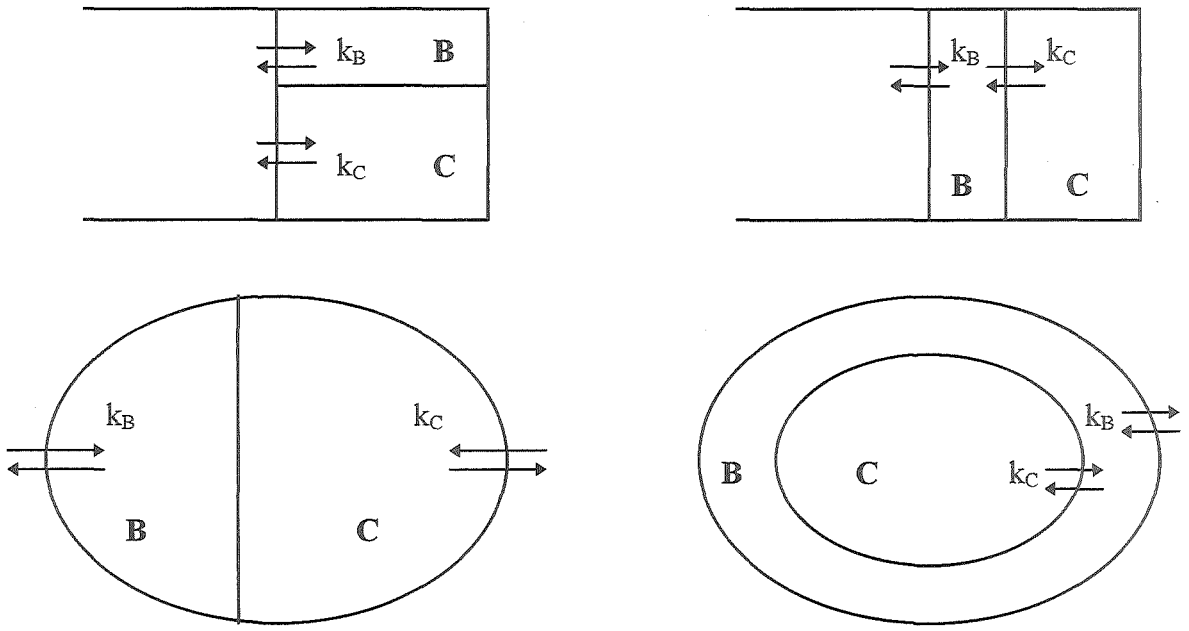

Figure 7.1 Two two-compartment models for exchange of water and sodium with seawater. Model 2 is considered more likely to represent crab eggs.

$$EWV_{TOTAL},\ Na_{TOTAL} = A_\infty / SA$$

$$EWV_B\ ,\ Na_B = B / SA$$

$$EWV_C\ ,\ Na_C = C / SA$$

where SA is the specific activity of the tracer in the medium (dpm.nL^{-1} or dpm.nmol^{-1} respectively).

For graphical presentation, tracer uptakes are expressed as the quantities of water (nL) or sodium (nmol) taken up per egg at time t (i.e. $A_t SA$).

Influxes into the inner pool were calculated as:

$$J_{influx(water)} = k_C \cdot C \qquad \text{nL.h}^{-1}$$

$$J_{influx(Na)} = k_C \cdot C \qquad \text{nmol.h}^{-1}$$

Permeabilities of the eggs to water and sodium were calculated as:

$$P = \frac{J_{influx}}{A \cdot M} \qquad \text{nL.h}^{-1}\text{.mm}^{-2}\text{.mol}^{-1}\text{.L}^{-1} \text{ or nmol.h}^{-1}\text{mm}^{-2}\text{mol}^{-1}\text{L}^{-1} \quad \text{respectively} \quad (7.3)$$

where A is the surface area of the egg (mm^2) and M is the molarity of water or sodium in the external medium.

According to equation 7.3, the unit nL.h^{-1}.mm^{-2}mol^{-1}.L^{-1} of water permeability was converted into mol.s^{-1}.cm^{-2}.mol^{-1}.cm^3 or cm.s^{-1}. This allowed all data to be directly compared with data obtained from other studies (Table 7.5).

The rate constants, influxes and permeabilities for pool B could not be accurately estimated, equilibrium for both water and sodium being established in less than 1 minute.

RESULTS

Water influx of developing eggs of H. rotundifrons

Water influx was determined with tritiated water in eggs at stage 2, 3, 4, 5A, 5C and 5D in 100% seawater, and in eggs at stage 2 and 5D in 50% seawater. The eggs of *H. rotundifrons* are very permeable to water at all stages of their development. Figure 7.2 and 7.3 show the relationship between the volume of water exchanged and time for eggs at stage 2 and 5D in 100% and 50% seawater. Water influx was resolved into a small rapidly exchanging pool and a much larger, slowly exchanging pool at every stage in both media. Calculated pool sizes, rate constants, flux and permeability data are presented for all stages in Table 7.1 and 7.2.

The total volume of exchangeable water (5 h equilibrium) of eggs at stage 2 was 90 nL in 100% seawater (Figure 7.2) Compartmental analysis resolved this into a "fast" pool of 14 nL and a "slow" pool of 77 nL. The rate constant for exchange of slow pool in 100% seawater was 2.17 h^{-1} ($T_{1/2}$= 0.319 h). In 50% seawater, the volume of the fast pool was halved (7 nL) and the slow pool increased by about 30% (105 nL). The rate constant of the slow pool was about half that in 100% seawater (significantly different, $P < 0.05$). As a percentage of total egg volume the fast and slow pools were about 6% and 33% respectively in 100% and 3% and 42% respectively in 50% seawater. Water influx rates and permeability for the slow pool of stage 2 eggs were not significantly different in 100% seawater and 50% seawater. Influx of water to the fast pool was exceedingly rapid in both media and could not be accurately estimated. Equilibrium was essentially complete by the first sample time (0.5 min).

In eggs at stage 5D, the total exchangeable water volumes at equilibrium were increased threefold; 286 and 335 nl in 100% and 50% seawater respectively. As in eggs at stage 2, there were two components of water exchange in both 100% and 50% seawater (Figure 7.3). The rate constant for the slow pool was significantly lower in 50% than in 100% seawater ($T_{1/2}$ = 1.08 h and 1.65 h respectively). There was no significant difference of exchangeable water volume and percentage of exchangeable water volume of eggs at this stage between 100% and 50% seawater of both the fast and slow pools. The water influx rates and permeability of the slow pool were significantly higher in 100% than in 50% seawater ($P < 0.05$).

Table 7.1 Mean ± SE of the exchangeable water volume, the rate constant, the rate of water influx and relative permeability in different egg stages of *Heterozius rotundifrons*, in 100 % sea water, temperature 20±1°C.

Stage	Total volume of egg (nL)	Surface area of egg (mm^2)	* Total exchangeable water volume (nL)	Rate constant (h^{-1})	Exchangeable water volume (nL)		Exchangeable water % Total volume		Water influx ($nL.h^{-1}$)	Water permeability ($nL.h^{-1}.mm^{-2}.mol^{-1}.L^{-1}$)	n
				inner pool	outer pool	inner pool	outer pool	inner pool	inner pool	inner pool	
2	231 ± 7	1.821	90 ± 4	2.17 ± 0.22	14 ± 1	77 ± 4	5.98 ± 0.42	33.42 ± 2.42	162 ± 1	1.64 ± 0.10	6
3	252 ± 10	1.930	166 ± 13	1.38 ± 0.08	18 ±1	148 ± 11	7.22 ± 0.51	58.64 ± 2.96	203 ± 16	1.93 ± 0.15	5
4	287 ± 1	2.103	176 ± 12	1.16 ± 0.05	22 ± 2	154 ± 13	7.66 ± 0.64	53.47 ± 4.44	177 ± 12	1.54 ± 0.11	5
5A	282 ± 4	2.080	172 ± 3	1.14 ± 0.04	18 ± 2	154 ± 4	6.23 ± 0.64	54.64 ± 0.93	175 ± 7	1.54 ± 0.06	5
5C	335 ± 8	2.333	245 ± 9	0.96 ± 0.05	16 ± 3	229 ± 8	4.79 ± 1.03	68.89 ± 3.28	218 ± 9	1.71 ± 0.07	6
5D	384 ± 8	2.555	286 ± 14	0.64 ± 0.04	14 ± 1	275 ± 11	3.68 ± 0.34	71.53 ± 2.30	176 ± 10	1.26 ± 0.07	6

* Total exchangeable water volume was determined at 5 h for stages 2-4, 5A, and for stage 5C-5D at 24 h. Outer and inner pools refer to fast and slow components of influx respectively (see Discussion and Methods sections).

Table 7.2 Mean ± SE of the exchangeable water volume, the rate constant, the rate of water influx and relative permeability in different egg stages of *Heterozius rotundifrons*, in 50 % sea water, temperature 20±1°C.

Stage	Total volume of egg (nL)	Surface area of egg (mm^2)	* Total exchangeable water volume (nL)	Rate constant (h^{-1})	Exchangeable water volume (nL)		Exchangeable water % Total volume		Water influx ($nL.h^{-1}$)	Water permeability ($nL.h^{-1}.mm^{-2}.mol^{-1}.L^{-1}$)	n
				inner pool	outer pool	inner pool	outer pool	inner pool	inner pool	inner pool	
2	253 ± 5	1.935	112 ± 4	1.33 ± 0.27	7 ± 0	105 ± 4	2.88 ± 0.21	41.66 ± 1.52	140 ± 28	1.31 ± 0.26	7
5D	405 ± 7	2.645	335 ± 11	0.42 ± 0.04	15 ± 2	320 ± 10	3.79 ± 0.43	78.81 ± 1.92	134 ± 10	0.92 ± 0.07	5

* Total exchangeable water volume was determined at 5 h for stages 2, and for stage 5D at 24 h, Outer and inner pools refer to fast and slow components of influx respectively (see Discussion and Methods sections).

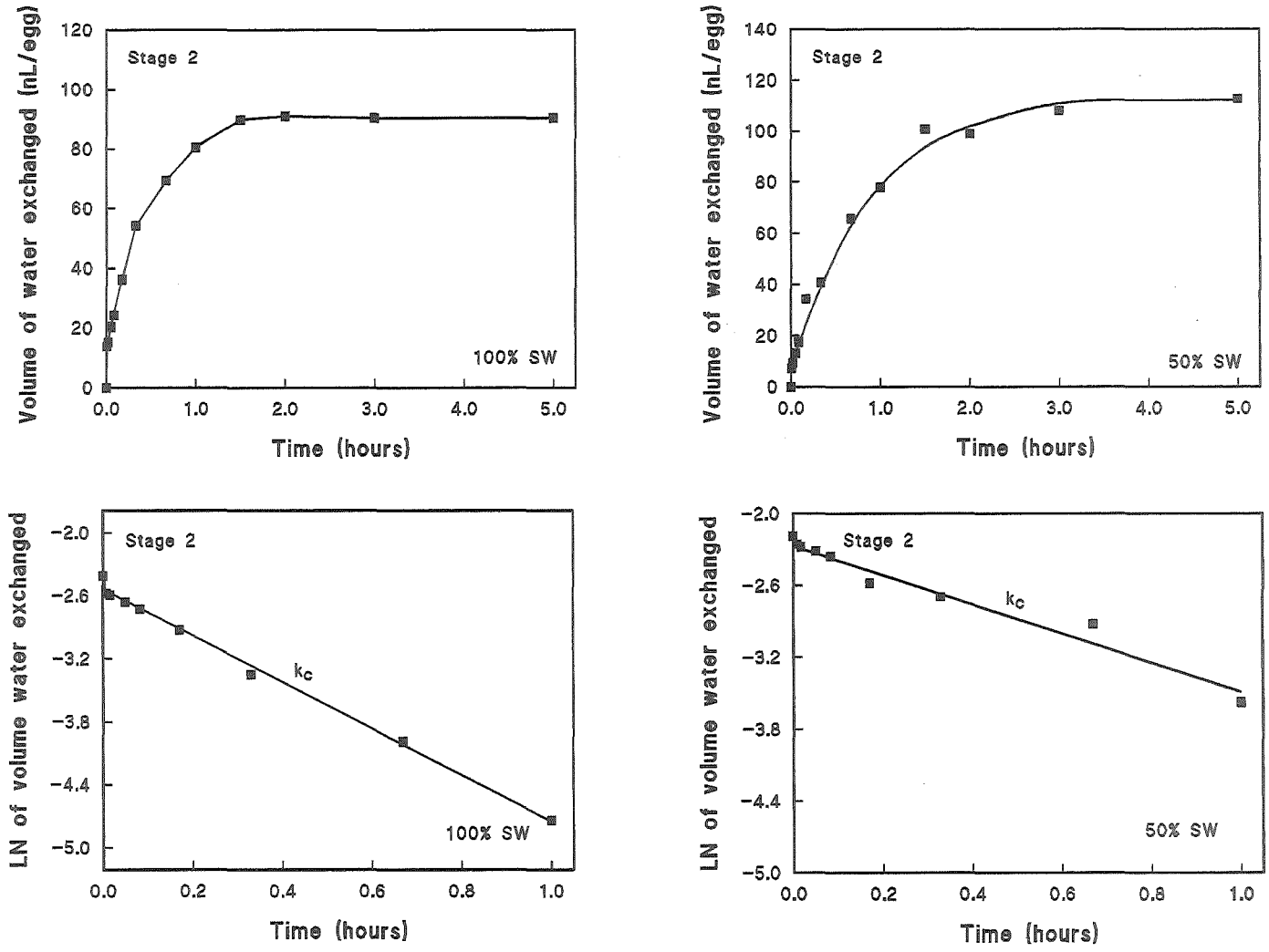

Figure 7.2 Exchangeable water volume of developing egg at stage 2 of *Heterozius rotundifrons* at successive times after placing in tritiated seawater at salinities 100% and 50%. Rate constant of water exchanged across the egg membrane for inner pool (k_c) in 100% and 50% seawater are 2.17 and 1.33 h^{-1} respectively. Each point is the mean of observations on 5-7 influx runs (detail in Table 7.1, 7.2)

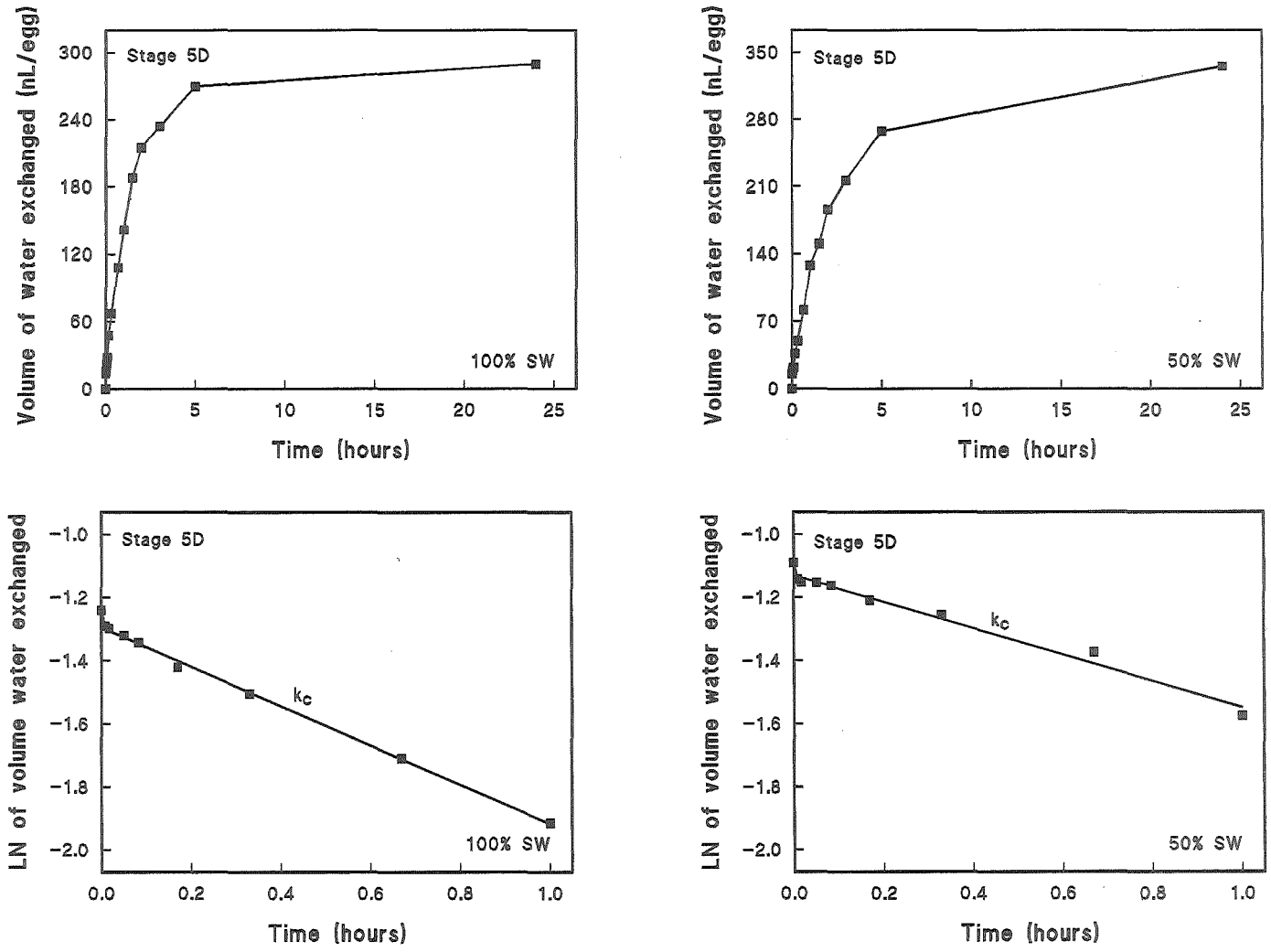

Figure 7.3 Exchangeable water volume of developing egg at stage 5D of *Heterozius rotundifrons* at successive times after placing in tritiated seawater at salinities 100% and 50%. Rate constant of water exchanged across the egg membrane for inner pool (k_c) in 100% and 50% seawater are 0.64 and 0.42 h^{-1} respectively. Each point is the mean of observations on 5-7 influx runs (detail in Table 7.1, 7.2)

The total exchangeable water volumes at equilibrium in 100% seawater of eggs at stage 3, 4, 5A and 5C were 166, 176, 172 and 245 nL respectively (Figure 7.4). As in eggs at stage 2 and 5D, there were two components of water exchange in eggs at these stages. The rate constant for the slow pool of egg at stage 3, 4, 5A and 5C ($T_{1/2}$ = 0.502, 0.597, 0.608 h and 0.722 h respectively) were significantly lower than eggs at stage 2 ($T_{1/2}$ = 0.319 h) and significantly higher than eggs at stage 5D ($T_{1/2}$ = 1.08 h). There was no significant difference of exchangeable water volume and percentage water volume of the fast pool between eggs at stage 2, 3, 4, 5A, 5C and 5D in 100% seawater The percentage of exchangeable water volumes of the slow pool of eggs at stage 3, 4, 5A were similar but they were significantly higher than eggs stage 2 and significantly lower than eggs at stage 5C and 5D. The water influx rates of the slow pool were significantly higher in eggs at stage 5C than stage 2 in 100% seawater ($P < 0.05$). The permeability of the slow pool were significantly higher in eggs at stage 3 than stage 5D in 100% seawater ($P < 0.05$).

Sodium influx of developing eggs of H. rotundifrons

Sodium influx was measured using ^{22}Na as a tracer in eggs at stages 2, 5B and 5D in 100% seawater, and in eggs at stage 2 and 5D in 50% seawater. The eggs of *H. rotundifrons* are very permeable to sodium at all stages of their development. Figure 7.5 and 7.6 show time course of sodium exchange in eggs at stage 2 and 5D in 100% and 50% seawater. Similar to water influx, sodium influx was resolved into a small rapidly exchanging pool and a much larger, slowly exchanging pool at every stage in both media. Calculated pool sizes, rate constants, flux and permeability data are presented for all stages in Table 7.3 and 7.4.

The total content of exchangeable sodium (5 h equilibrium) of eggs at stage 2 was 14.03 nmol in 100% seawater (Figure 7.5, Table 7.3). The exchangeable sodium of the fast pool and the slow pool were 9.01 and 5.02 nmol respectively. The rate constant for exchange of the slow pool in 100% seawater was 0.47 h^{-1} ($T_{1/2}$ = 1.47 h). In 50% seawater, the content of sodium of the fast pool and the slow pool decreased by about 56% and 64% (3.99 and 1.81 nmol respectively). There was no significant difference of the rate constant between 100% and 50% seawater of the slow pool. Sodium influx rate and permeability for the slow pool were higher in 100% than in 50% seawater (significantly different, $P < 0.05$). Influx of sodium to the fast pool was exceedingly rapid in both media and could not be accurately estimated.

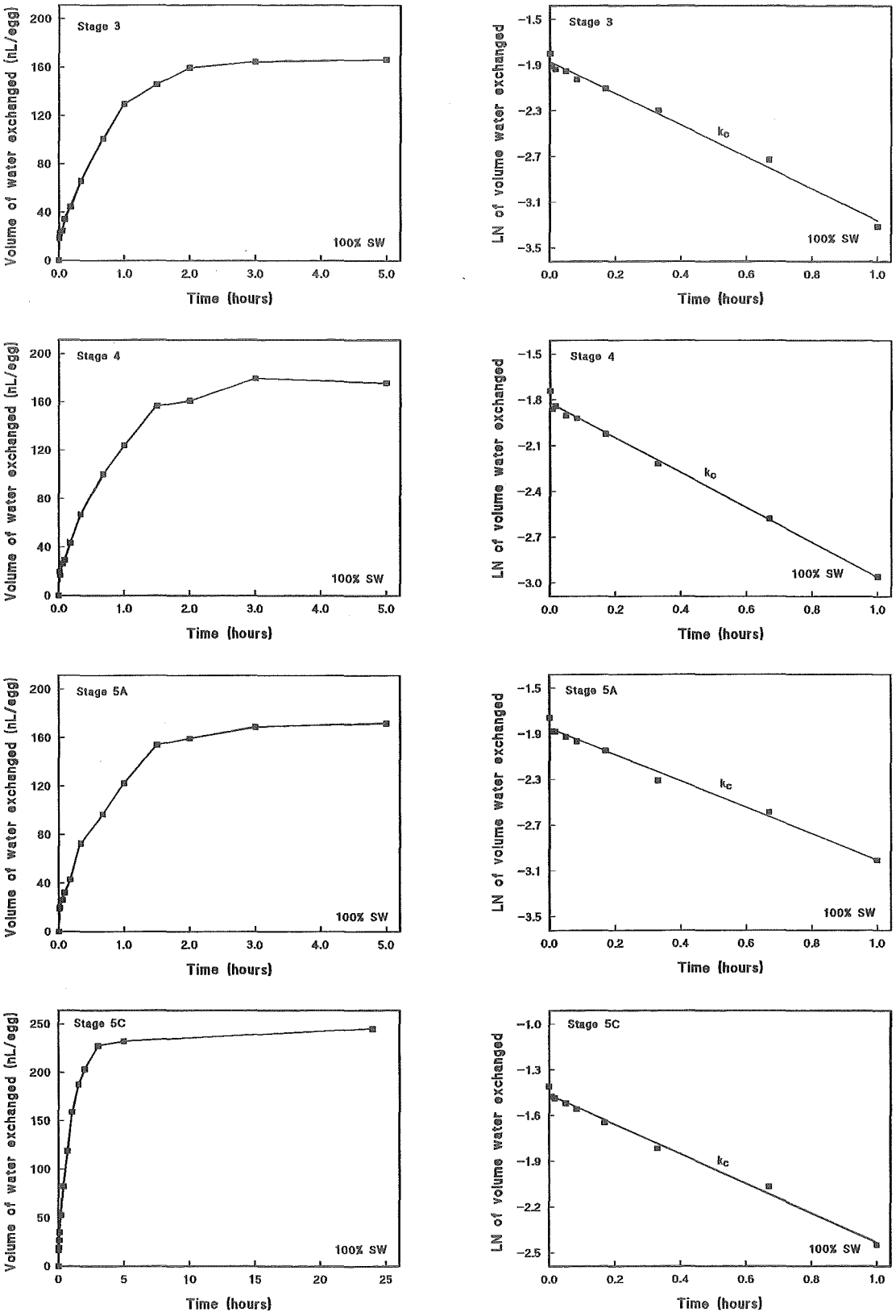

Figure 7.4 Exchangeable water volume of developing eggs at stage 3, 4, 5A and 5C of *Heterozius rotundifrons* at successive times after placing in tritiated seawater at salinity 100%. Rate constant of water exchanged across the egg membrane for inner pool (k_C) of eggs at stage 3, 4, 5A and 5C are 1.38, 1.16, 1.14 and 0.96 h^{-1} respectively. Each point is the mean of observations on 5-6 influx runs (detail in Table 7.1)

Table 7.3 Mean ± SE of the exchangeable sodium, the rate constant and the rate of sodium influx in different egg stages of *Heterozius rotundifrons*, in 100 % sea water, temperature 20±1°C.

Stage	Total volume of egg (nL)	Surface area of egg (mm^2)	*Total sodium in one egg (nmol)	**Total exchangeable sodium in one egg (nmol)	Rate constant (h^{-1})	Exchangeable sodium (nmol)		Sodium influx ($nmol.h^{-1}$)	Sodium permeability ($nmol.h^{-1}.mm^{-2}.mol^{-1}.L^{-1}$)	n
					inner pool	outer pool	inner pool	inner pool	inner pool	
2	241± 6	1.873	18.8	14.03 ± 0.29	0.47 ± 0.07	9.01 ± 0.32	5.02 ± 0.38	2.38 ± 0.35	2.81 ± 0.41	6
5B	290 ± 7	2.124	28.61	30.33 ± 0.94	0.08 ± 0.01	8.28 ± 0.33	22.04 ± 1.00	1.72 ± 0.18	1.80 ± 0.19	4
5D	370 ± 8	2.491	76.42	96.28 ± 2.90	0.11 ± 0.02	15.19 ± 3.13	81.09 ± 0.38	8.92 ± 1.30	7.94 ± 1.16	3

* Total sodium was determined by atomic absorption (chapter 6)
** The exchangeable sodium was determined at 5 h for stage 2, and for stage 5B and 5D at 24 h
Outer and inner pools refer to fast and slow components of influx respectively (see Discussion and Methods sections).

Table 7.4 Mean ± SE of the exchangeable sodium, the rate constant and the rate of sodium influx in different egg stages of *Heterozius rotundifrons*, in 50 % sea water, temperature 20±1°C.

Stage	Total volume of egg (nL)	Surface area of egg (mm^2)	*Total sodium in one egg (nmol)	**Total exchangeable sodium in one egg (nmol)	Rate constant (h^{-1})	Exchangeable sodium (nmol)		Sodium influx ($nmol.h^{-1}$)	Sodium Permeability ($nmol.h^{-1}.mm^{-2}.mol^{-1}.L^{-1}$)	n
					inner pool	outer pool	inner pool	inner pool	inner pool	
2	255 ± 4	1.942	5.60	5.80 ± 0.18	0.43 ± 0.06	3.99 ± 0.22	1.81 ± 0.17	0.81 ± 0.13	1.78 ± 0.28	7
5D	403 ± 7	2.638	58.72	48.12 ± 6.41	0.05 ± 0.01	7.83 ± 0.83	40.30 ± 6.47	1.81 ± 0.27	2.93 ± 0.43	4

* Total sodium was determined by atomic absorption (chapter 6)
** The exchangeable sodium was determined at 5 h for stage 2, and for stage 5D at 24 h
Outer and inner pools refer to fast and slow components of influx respectively (see Discussion and Methods sections).

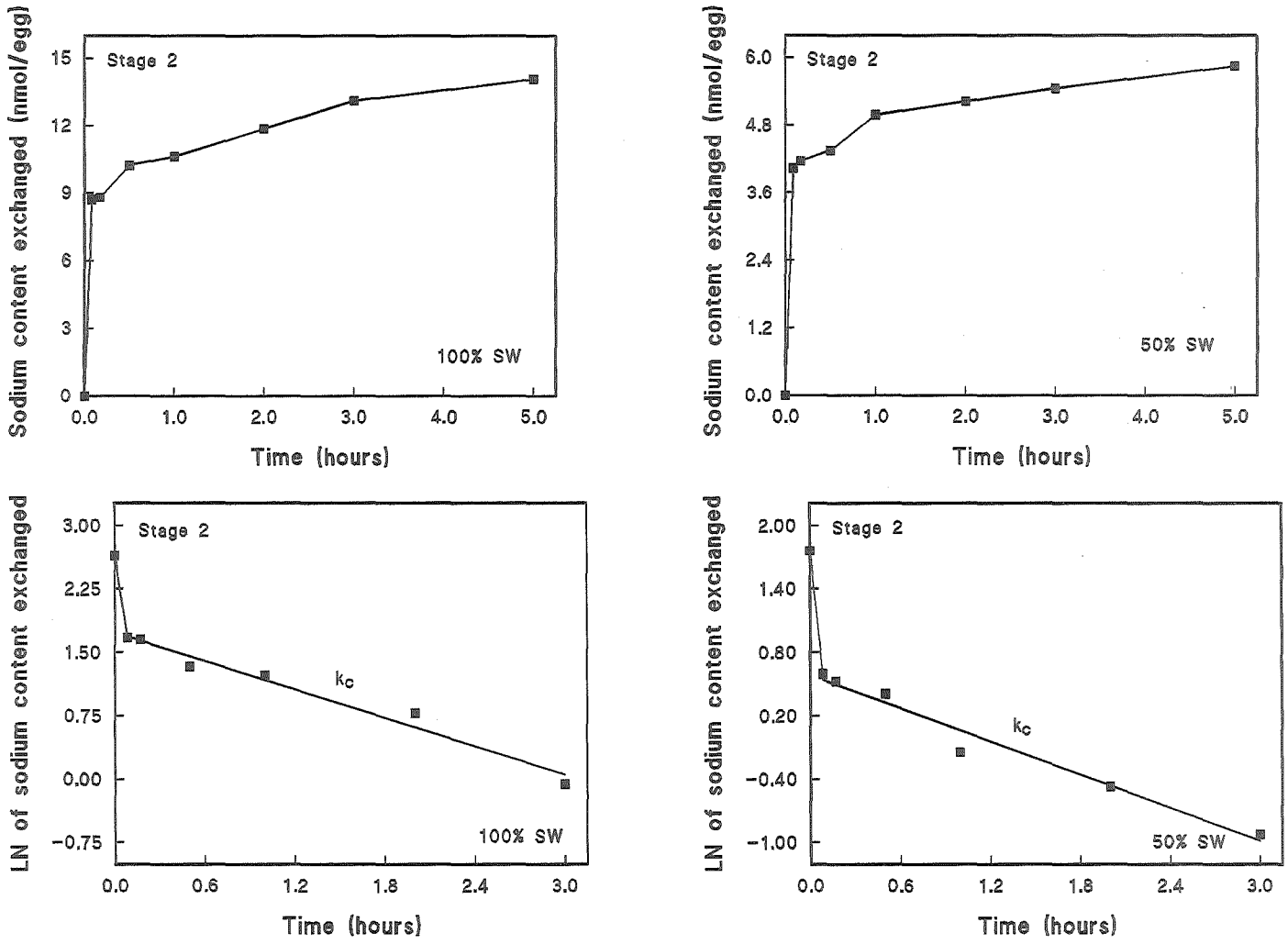

Figure 7.5 Exchangeable sodium content of developing egg at stage 2 of *Heterozius rotundifrons* at successive times after placing in ^{22}Na-labelled seawater at salinities 100% and 50%. Rate constant of sodium across the egg membrane for inner pool (k_C) in 100% and 50% seawater are 0.47 and 0.43 h^{-1} respectively. Each point is the mean of observations on 6-7 influx runs (detail in Table 7.3, 7.4)

In eggs at stage 5D, the total exchangeable sodium contents after 24 h were increased about 7-8 fold from stage 2 (96.28 and 48.12 nmol in 100% and 50% seawater respectively) (Figure 7.6). In 50% seawater, however, ^{22}Na content appeared to be still increasing at 24 h. Thus, the exchangeable sodium of the slow pool may be underestimated, and the rate constant overestimated, in this case. The rate constant for the slow pool in 50% was about half that in 100% seawater ($T_{1/2}$ = 13.86 h and 6.3 h) (significantly different $P < 0.05$). The exchangeable sodium of both fast and slow pools in 50% seawater were about half that in 100% seawater (significantly different $P < 0.05$). The sodium influx and permeability of the slow pool were about 5-fold and 3-fold higher in 100% than in 50% seawater (Note: these values would not be affected by lack of equilibrium at 24 h because they are based on only the first 3 hours).

In eggs at stage 5B, the total exchangeable sodium contents after 24 h was 30.33 nmol in 100% seawater (Figure 7.7). As for eggs at stage 2 and 5D, there were two components of sodium exchange in eggs at this stage. The rate constant of the slow pool ($T_{1/2}$ = 8.7 h^{-1}) was about 6-fold lower than that in eggs at stage 2. The exchangeable sodium contents of the fast pool was similar to eggs at stage 2, but it was about half that of eggs at stage 5D in 100% seawater. There were significant difference of exchangeable sodium for the slow pool between eggs at this stage, stage 2 and stage 5D ($P < 0.05$). The sodium influx rates and permeability of the slow pool were similar between eggs at this stage and stage 2, but were significantly lower than stage 5D ($P < 0.05$).

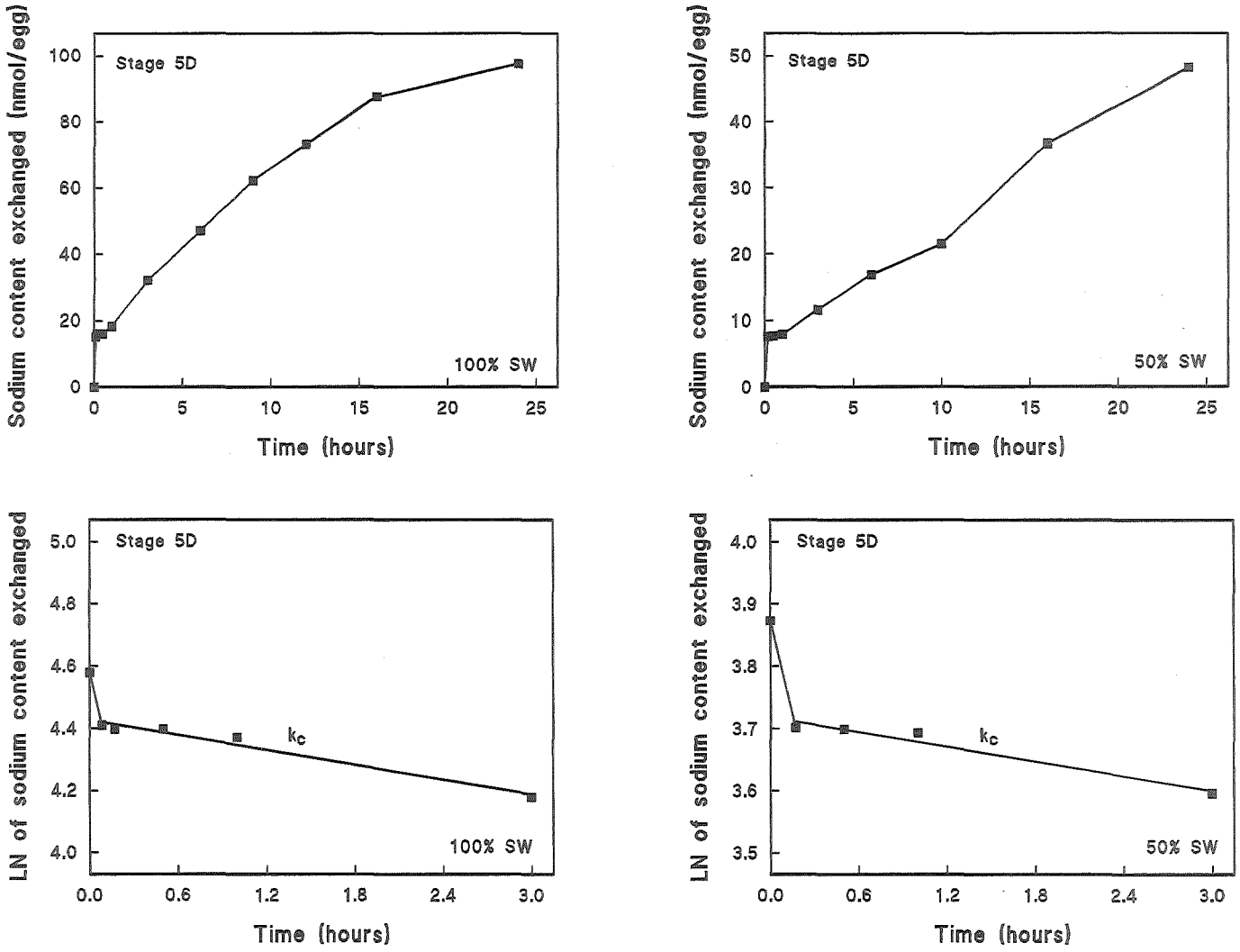

Figure 7.6 Exchangeable sodium content of developing eggs at stage 5D of *Heterozius rotundifrons* at successive times after placing in ^{22}Na-labelled seawater at salinities 100% and 50%. Rate constant of sodium across the egg membrane for inner pool (k_C) in 100% and 50% seawater are 0.11 and 0.05 h^{-1} respectively. Each point is the mean of observations on 3-4 influx runs (detail in Table 7.3, 7.4)

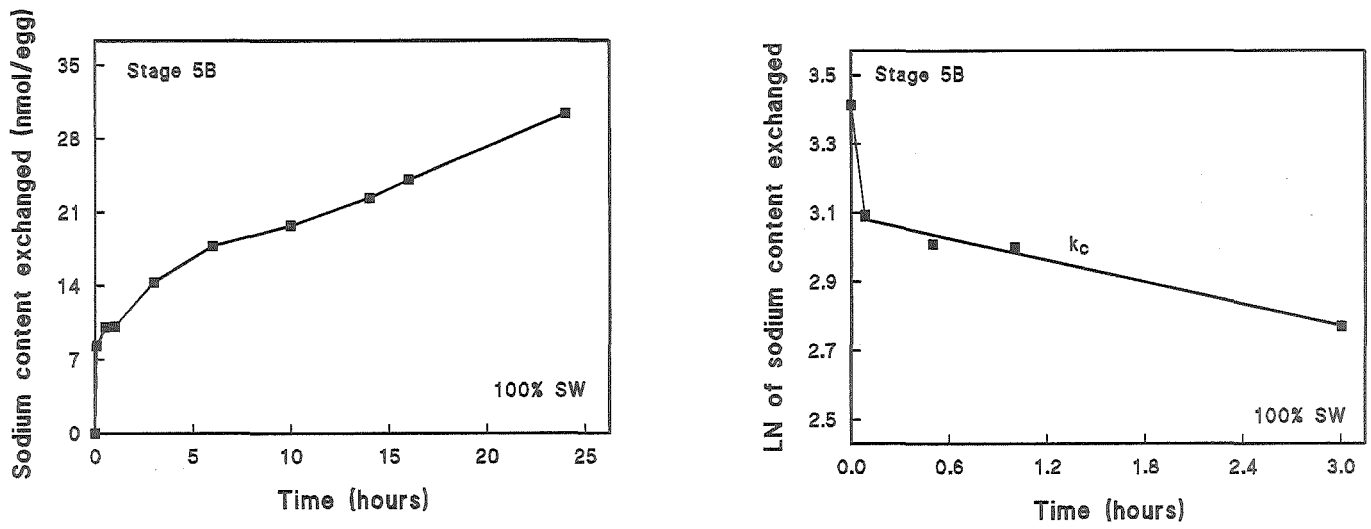

Figure 7.7 Exchangeable sodium content of developing eggs at stage 5B of *Heterozius rotundifrons* at successive times after placing in ^{22}Na-labelled seawater at salinities 100%. Rate constant of sodium across the egg membrane for inner pool (k_c) are 0.08 h^{-1}. Each point is the mean of observations on 4 influx runs (detail in Table 7.3)

DISCUSSION

Pandian (1970a) studied quantitative changes in salt (ash) and water contents in *Homarus gammarus* eggs. He concluded that initial high permeability of the egg membrane to water is followed by a period during which the egg membrane is almost water impermeable (Stage I to III); the egg membrane then becomes permeable to water again at a relatively advanced stage of development (from stage III on). Unlike *H. gammarus*, the results of experiment on water influx suggested that egg membrane of *H. rotundifrons* are very permeable to water and salt throughout their development, although there were some changes in membrane permeability. However, Pandian's method is based on total water content rather than fluxes and cannot provide unequivocal information on permeability.

There were two components of water and sodium exchange with the seawater in all egg stages of *H. rotundifrons*; a rapidly exchanging pool and a slowly exchanging pool. There is no morphological basis for two independently exchanging pools (Figure 7.1, model 1). Thus, the most likely configuration is a sequential or concentric model (Figure 7.1, model 2) in which the fast pool is on the outside. Morphological studies (chapter 2) suggested two membranes (the outer and the inner membranes) surrounding the developing eggs of *H. rotundifrons*. Thus, it is possible that exchange with the fast pool occurred through the outer membrane, between the external medium and the perivitelline space of eggs, or between the external medium and the egg membranes themselves. Exchange with the slow pool is thus envisaged as occurring through the inner membrane between the perivitelline space and the embryo and/or yolk inside. In late stage eggs with well-developed embryos inside, there might be more than two membranes (i.e. cuticle of the embryos and membranes of some tissues) which would suggest more than two compartments. However, the method used in this study could not detect these compartments.

Water influx study showed that total exchangeable water volume of eggs increased with their development. This was correlated with the increase in total volume (chapter 2) and solvent volume (chapter 6) of eggs with their development. There was a good correspondence between the total exchangeable water volume and the solvent volume of *H. rotundifrons* eggs. The total exchangeable water volume and the solvent volume of eggs at stage 2 were 90 and 118 nL and those of stage 5A were 172 and 176 nL respectively in 100% seawater. As noted, there were two pools of exchangeable water. The volume of the outer pool was less than the inner pool and as a percentage of total

volume it changed very little with egg development compared with inner pool. As already noted, it seems likely that the outer pool represents the perivitelline space of eggs. Its volume of about 4-8% throughout development is consistent with morphological appearance (chapter 2). Thus, there is not much change in the size of perivitelline space from the beginning to the end of development of eggs. In contrast, there was an increase in percentage of exchangeable water volume throughout development for the inner pool. The percentage of exchangeable volume water of the inner pool of developing egg at stage 2 was about 33% of the whole egg volume. At stage 3, the percentage of exchangeable water volume increased to about 59% of the whole egg volume and became constant during stage 4 and 5A. Finally, the exchangeable water volume reached its maximum value at stage 5C and 5D, with the percentage exchangeable volume about 70% of the total volume. This corresponds with observations by Pandian (1970a) on *Homarus gammarus*. He reported that during the course of its embryonic development, the European lobster *H. gammarus* exhibited progressive increases in content of water (54.0% in fresh eggs to 83.1% in larvae). Similar data are also reported in marine demersal eggs of crustaceans and cephalopods that they increased their water content from about 50-60% to about 70-80% during embryonic development (Pandian, 1970a). The embryonic development study (chapter 2) found that during development, the quantity of yolk decreased with development. Thus, it is possible that during development, yolk was used up and replaced by water. Additionally, the whole egg swells during development as the egg volume increased from about 204 nL at the blastula stage to about 361 nL at the last stage (chapter 2).

From the previous study (chapter 6), eggs at the early stages might be expected to have more permeable membranes to water, compared with the last stage since the early stage eggs had reduced abilities to survive in diluted seawater. In agreement with this prediction, it was found that the absolute membrane permeabilities of eggs at stage 2, 3, 4, 5A, 5C were higher than that of stage 5D. In lobster eggs, Pandian (1970a) suggested that the possible cause for sudden change in egg membrane permeability to water is that there is a special hatching enzyme whose function is the chemical alteration of the egg membrane. It is possible that the changes in the permeability of the membrane of crab eggs in this study is due to the differing amounts of lipoids or other chemical substances in the membrane.

Table 7.5 compares the water permeability of egg membranes of *H. rotundifrons* eggs and a number of cells and tissues of animals including synthetic membranes. *H. rotundifrons* eggs in both 100% and 50% seawater have lower water permeability than other tissues and synthetic membranes. This suggested that the surface membrane of

Table 7.5 Comparisons of diffusion permeability between *Heterozius rotundifrons* eggs (in 100% and 50% seawater) and a number of cells and tissues of animals including synthetic membranes.

Cells or tissues or membranes	Water permeability ($cm.sec^{-1}$)	References
*Amoeba	2.3×10^{-5}	Prescott and Zeuthen (1953)
*Frog, ovarian egg	1.34×10^{-4}	Prescott and Zeuthen (1953)
*Frog, body cavity egg	7.5×10^{-5}	Prescott and Zeuthen (1953)
*Xenopus, body cavity egg	9.0×10^{-5}	Prescott and Zeuthen (1953)
*Zebra fish, ovarian egg	6.8×10^{-5}	Prescott and Zeuthen (1953)
*Zebra fish, shed egg	3.6×10^{-5}	Prescott and Zeuthen (1953)
**Salmo salar*, egg	1.0×10^{-6}	Loeffler *et al.* (1970)
**Fundulus* sp., egg	1.0×10^{-6}	Dunham *et al.* (1970)
**Esox lucius*, egg	1.8×10^{-6}	Loeffler (1971)
**Pleuronectes* sp., egg	1.7×10^{-7}	Potts and Eddy (1973)
Gadus morhua, unfertilized egg	8.5×10^{-6}	Mangor-Jensen (1987)
Gadus morhua, fertilized egg	1.78×10^{-7}	Mangor-Jensen (1987)
*Human (adult) erythrocyte	5.3×10^{-3}	Sidel & Solomon (1957); Paganelli & Solomon (1957)
*Human (fetal) erythrocyte	2.3×10^{-3}	Sjölin (1954); Barton & Brown (1964)
*Squid axon	1.4×10^{-4}	Villegas & Villegas (1960)
*Crab muscle	1.2×10^{-4}	Sorenson (1971)
*Barnacle muscle	2.6×10^{-4}	Bunch & Edwards (1969)
*Toad bladder: No vasopressin	9.5×10^{-5}	Hays & Leaf (1962)
*Toad bladder: With vasopressin	1.6×10^{-4}	Hays & Leaf (1962)
*Lipid	4.4×10^{-4}	Huang & Thompson (1966)
*Cellophane	4.0×10^{-4}	Durbin (1960)
*Dialysis	1.09×10^{-3}	Ginzburg & Katchalsky (1963)
*Wet gel	1.92×10^{-3}	Ginzburg & Katchalsky (1963)
***H. rotundifrons* eggs (stage 2)	2.48×10^{-6}	Present study
***H. rotundifrons* eggs (stage 3)	2.92×10^{-6}	Present study
***H. rotundifrons* eggs (stage 4)	2.33×10^{-6}	Present study
***H. rotundifrons* eggs (stage5A)	2.33×10^{-6}	Present study
***H. rotundifrons* eggs (stage 5C)	2.59×10^{-6}	Present study
***H. rotundifrons* eggs (stage 5D)	1.91×10^{-6}	Present study
****H. rotundifrons* eggs (stage 2)	1.98×10^{-6}	Present study
****H. rotundifrons* eggs (stage 5D)	1.39×10^{-6}	Present study

*Data are reobtained from Stein (1967), House (1973) and Mangor-Jensen (1987).
** and *** represent eggs in 100% and 50% seawater respectively

crab egg differs from other tissues and synthetic membranes. When compared with other egg cells, water permeability of *H. rotundifrons* eggs are in the range between these cells. Compared with some marine fish eggs, water permeabilities of *H. rotundifrons* eggs were found to be higher. This suggests an evolutionary adaptation of this crab to live in the intertidal zone.

Eggs at stage 5D acclimated to 50% seawater showed absolute permeability of the membrane less than eggs acclimated to 100% seawater. The results of this study indicated that developing eggs at this stages can reduce their water exchange or the permeability of their membrane upon acclimation to a lower salinity. Cantelmo (1977) studied water permeability of isolated tissues of crabs, found that osmotically active tissues such as the gill and gut of crabs exhibit a reduction in permeability when exposed to a lower salinity. Smith (1967) and Capen (1972) also reported that in *R. harrisi* there is a significant lowering in apparent permeability to water of the whole crab after they are acclimated to lower salinities. Smith (1970) found that *C. maenas* shows a reduction in apparent permeability in 50% seawater. Smith & Rudy (1972) stated that *H. nudus* also shows a reduction when acclimated to 60% seawater. Thus, reduction of water permeability upon acclimation to a lower salinity appears to be a generalized response that is also part of the total osmotic response of crab eggs to a change in salinity. This mechanism is of obvious advantage to developing eggs that inhabit an intertidal environment because such a reduction in input of water would limit the work necessary to balance osmotic influx of water.

Sodium influx study showed that total exchangeable sodium of eggs increased with their development. The total exchangeable sodium of eggs at stage 2 and 5D were in equilibrium after 5 and 24 hours respectively in 100% seawater. In eggs at stage 5D, the total exchangeable sodium measured at equilibrium in 100% seawater was significantly higher than the total sodium contents measured by AAS method. The difference could possibly due to either underestimating the total sodium contents by AAS method or overestimating the exchangeable sodium or actual differences between the ion content of the egg batches caused by staging differences. There were two pools of exchangeable sodium found in eggs. The exchangeable sodium content of the outer pool was more than the inner pool in eggs at stage 2, but it became less than the inner pool at the later stage. The size of exchangeable water volume of the outer pool did not change with egg development compared with that of the inner pool. However, the total sodium contents of this outer pool changed from 9.01 nmol in eggs at stage 2 to 15.19 nmol in eggs at stage 5D. From the estimation of the exchangeable water and sodium contents of the outer pool, it is possible to calculate the sodium concentration in the

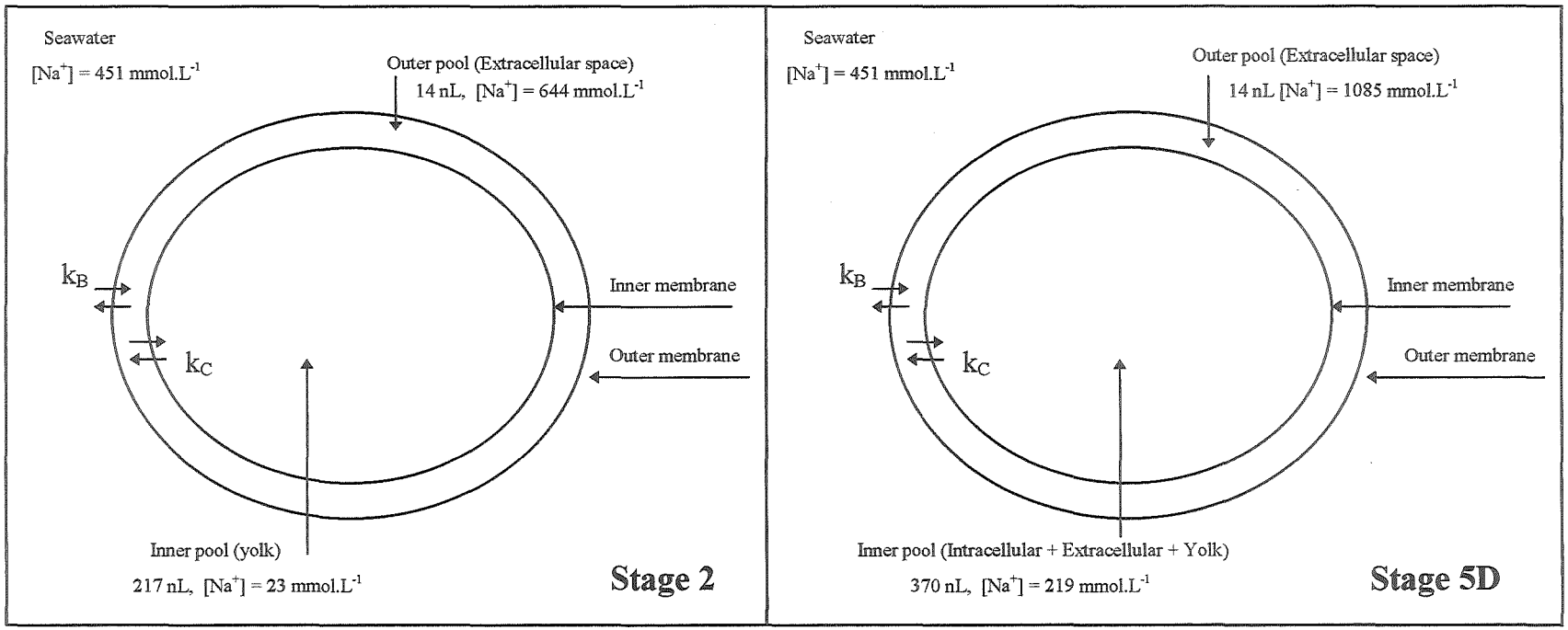

Figure 7.8 Models for distribution of water and sodium in eggs at stage 2 and 5D of *Heterozius rotundifrons*. Water and sodium are exchanged as two-compartment systems similar to Model 2 in Figure 7.1.

perivitelline space (and/or membranes) of eggs. Figure 7.8 shows a simple model which is consistent with model 2 of the two-compartment model in Figure 7.1. The main features of the model are that there are two main sodium pools in the embryos. The outer pool is located in the perivitelline space and the inner pool represents the whole area located within the inner membrane. The perivitelline spaces of eggs at stage 2 and 5D have higher sodium concentrations than seawater (644 and 1085 $mmol.L^{-1}$ respectively; c.f. seawater 451 $mmol.L^{-1}$). The inner pool of eggs at stage 2 has a very low sodium concentration (23 $mmol.L^{-1}$) and it increased about 10 times in eggs at stage 5D (219 $mmol.L^{-1}$). This model, however, is unlikely to be true since the concentration of sodium in the perivitelline spaces especially of the late stage eggs was very much higher than those in the seawater.

The total exchangeable sodium of eggs at stage 2 and 5D were measured after 5 and 24 hours respectively in 50% seawater. In eggs at stage 5D, however, ^{22}Na appeared to be still increasing at 24 hours. Thus, in this case, the slow sodium pool size may be underestimated, and the rate constants overestimated. However, influx and permeability estimates, based on data from the first 3 hours are relatively unaffected by a lack of equilibrium at 24 hours. The influx rate of sodium of the inner pool in eggs at stage 2 and 5D acclimated to 50% seawater decreased to about a half and one-fourth of those found in 100% seawater. Salinity effects on ion flux activity has been studied on the gill of some crabs. Lucu and Siebers (1986) found that acclimation of *Carcinus mediterraneus* to dilute environment results in a considerable decrease in the magnitude of Na^+ fluxes across isolated perfused gills. Reduction in the permeability of egg membrane to sodium upon acclimation to a lower salinity appears to be a mechanism for maintenance of osmotic concentration within the egg cell relative to the outside medium. This mechanism is of obvious advantage to developing eggs of intertidal inhabitants because such a reduction in the rate of sodium influx would enable the rate of sodium exchange and the energy expended on sodium regulation to be reduced.

In conclusion, water and sodium influx studies showed that eggs of *H. rotundifrons* are very permeable to water and sodium throughout their development. Thus, crab eggs did not develop a closed system with an impermeable barrier to the passage of water or ions across the surface. Instead, they exist in a dynamic steady state and have abilities of hyperosmotic regulation in dilute seawater. It therefore appears that the eggs in dilute seawater must be performing of osmotic work involving active uptake of ions and active excretion of water. The routes for return of water and sodium to the medium clearly require further investigation.

CHAPTER EIGHT

CONCLUSION

Heterozius rotundifrons and *Cyclograpsus lavauxi* are intertidal crabs found at different positions on the shore of New Zealand. The aim of this thesis was to study the ecological and physiological adaptations of ovigerous crabs and their developing eggs to conditions on the shore comparing these two crab species.

Like other marine crabs, ovigerous females of both *H. rotundifrons* and *C. lavauxi* incubate their fertilized eggs by attaching them to the abdominal appendages. Although both crabs have similar size (carapace width are in the range of 20-25 mm), their eggs have different size. Mean volume of both early and late stage eggs of *H. rotundifrons* is about 20 times more than those of *C. lavauxi.* Mean diameters of eggs of *H. rotundifrons* and *C. lavauxi* increased 17-22% and 20-24% respectively from newly-laid eggs to the stage before hatching (mean volume of eggs increased about 77% and 84% in *H. rotundifrons* and *C. lavauxi* respectively). Wear (1974) suggested that among decapod crustaceans which are closely related, increasing egg size slows down the rate of development. He also suggested that during embryonic development, the rate of egg volume increase is considered slower in the eggs of species with a long development period than in those which develop rapidly and the rate of increase of egg volume accelerated during development, especially in the latter stage. This corresponds to the present study which found that the rate of egg volume increase was slower in the eggs of *H. rotundifrons* than those of *C. lavauxi.* The incubation periods of *H. rotundifrons* and *C. lavauxi* were 194±3 and 56±1 days at 15°C respectively. In the field, however, the incubation period for *H. rotundifrons* eggs might be longer since newly spawned eggs were found in late-March and the last stage eggs were found during mid-December. It is not surprising for such a long incubation period found in this species since average temperature at Kaikoura ranged from 8°C to 18°C (Rasmussen, 1965). The difference in the incubation time between eggs developed at constant 15°C and in natural condition (at Kaikoura) of *H. rotundifrons* suggested a form of diapause. In other decapods, however, diapause period was found in eggs at gastrula stage and could not be shortened significantly by raising the water temperature (Wear, 1974). Thus, it is possible that the difference in incubation time of eggs in this crab is not concerned with the diapause period but being affected directly by the difference in temperature. This interesting possibility requires further investigation.

Embryonic studies revealed that there were changes in morphology and physiology of eggs throughout their development in both crab species. By using the similar criteria (such as the formation of blastula and gastrula, the appearance of eye spots and heartbeats) used for characterizing the embryonic stages of other decapods (Morris, 1975; Amsler & George, 1984; Subramonian, 1979), eggs of both crab species can be conveniently defined into 5 main stages: (1) Newly laid eggs and cleavage stage (2) Morula and blastula stage (3) Gastrula and germ layer stage (4) Eyespots and pigmentation stage and (5) Heart-beating stage. There was no difference in pattern of egg development between these two species. However, time course of egg volume change during development was different between *H. rotundifrons* and *C. lavauxi*. In *H. rotundifrons,* egg volumes were relatively constant at blastula stage and slightly increased after gastrulation. There was a rapid increase in the volumes of eggs from about the time the embryonic heartbeat was first observable. In *C. lavauxi,* however, this more rapid increase in volume occurred soon after gastrulation. As in other decapods (Wear, 1974), the rate of yolk metabolism of both species was slower during early development than during the last period before hatching. Characters such as chromatophores, black eye pigment and the first indications of heartbeat of *H. rotundifrons* appeared with about 20% of the original yolk volume metabolized compared with about 60% in eggs of *C. lavauxi.* Likewise, in *Maia squinado* which has very large eggs (0.23 mm^3), these characters appeared with about 24% of the original yolk volume metabolized compared with about 60% in the very small eggs (0.013 mm^3) of *Macropipus spp.* (Wear, 1974).

Respiratory physiology has been studied in ovigerous crabs and their developing eggs. Both early and late-ovigerous *H. rotundifrons* and *C. lavauxi* consumed more oxygen than non-ovigerous crabs in both air and water (15°C). $\dot{M}o_2$ in water of early-ovigerous crabs was less than that of the late-ovigerous crabs in both species. This was correlated with the data on oxygen consumption in water of developing eggs. In both species, $\dot{M}o_2$ increased as development of eggs progressed. In blastula and gastrula stages, oxygen consumption was extremely low and, on a mass specific basis, was about the same as the adult crabs, despite being lower in mass by factors of more than 10^4 (*H. rotundifrons*) and 10^6 (*C. lavauxi*). These low rates are presumably partly attributed to the relatively large proportion of the cell occupied by yolk. As development progressed, the proportion of active cytoplasm increased and respiration increased 12 and 10 times in *H. rotundifrons* and *C lavauxi* respectively. Thus, despite differences in mass by factor of 20, the mass-specific oxygen consumptions were closely similar in the two species.

Whether the difference in respiratory rates between non-ovigerous and ovigerous crabs is due to the increased oxygen consumed by developing eggs or the increased energy for carrying broods was clarified by subtracting the calculated total oxygen consumption of the whole egg mass from oxygen consumption of ovigerous females. It was found that after substraction of oxygen uptake by their broods, the metabolic rates of ovigerous females without their embryos (both early and late stages) of both species of crabs were only slightly higher than that of non-ovigerous crabs. Thus, the increased metabolism of ovigerous crabs must mainly be due to the respiration of their embryos.

The metabolic cost of development, in term of oxygen consumption, of single eggs of *H. rotundifrons* and *C. lavauxi* were 1.517 and 0.077 μmol O_2 respectively. The total cost of development of an egg of *C. lavauxi* is about 20 times less than of *H. rotundifrons*. The 20-fold ratio is approximately equal to the ratio of their masses (about 19 times) which in turn reflects the trophic investment by the parent in each egg. The longer development time of *H. rotundifrons* than *C. lavauxi* is reflected in the slower rise in oxygen consumption in the first species. There is a correlation between the time course of changes in $\dot{M}O_2$ in eggs and the timing of disappearance of yolk. In both species, when the rate of oxygen consumption increased, the rate of disappearance of yolk also increased.

The oxygen consumption of *H. rotundifrons* eggs declined progressively as the Po_2 of the water was reduced, i.e. under these conditions the eggs exhibited a low level of respiratory independence. In contrast, oxygen consumption of eggs of *C. lavauxi* was decreased by only 30% down to about 45 mmHg below which the oxygen consumption of the eggs was strongly attenuated by further reduction in Po_2. This suggested that eggs of *H. rotundifrons* and *C. lavauxi* exhibit characteristics of oxygen conformer and regulator respectively. In adults, the respiration of aquatic animals is generally more sensitive to oxygen level than terrestrial animals (Vernberg & Vernberg, 1983). Thus, the higher O_2 sensitivity of eggs of the low-shore *H. rotundifrons* than the high-shore *C. lavauxi* is consistent with this trend. However, the direct difference in pattern of oxygen consumption between eggs of *H. rotundifrons* and *C. lavauxi* is perhaps more directly due to the difference in the surface area for gas exchange. Eggs of *C. lavauxi* have a sufficiently large surface area in relation to their volume to be able to take in all the oxygen they need by diffusion across their surface, whereas, in eggs of *H. rotundifrons* the lower respiratory surface area per unit mass is much lower and may limit oxygen uptake at low Po_2. As the later stage embryos used in the hypoxia experiments had partially developed circulation systems and exhibited some movements, it is possible that differences in convection of embryonic fluids also contribute to the

different response to hypoxia of the two species. This possibility requires further investigation.

When experiencing hypoxia, animals may switch from aerobic to anaerobic metabolism. Lactate accumulation found in eggs of *H. rotundifrons* has proved that crab eggs also can switch from aerobic to anaerobic metabolism upon exposure to hypoxia. Lactate concentration in *H. rotundifrons* eggs increased from about 0.602 $mmol.L^{-1}$ in normoxic eggs to about 10.20 $mmol.L^{-1}$ after three hours period of hypoxic exposure. However, the ATP energy production obtained from lactate is about 18 times less than those from O_2 uptake. Clearly, during hypoxia, the eggs are not sustaining metabolism by anaerobiosis, but are allowing metabolic depression. Presumably, much of the energy deficit can be recovered on return to normoxia by remetabolising the lactate. However, regular exposure to hypoxia would be expected to delay development in eggs of this species.

Since the eggs are not fully sustaining metabolism by anaerobiosis during hypoxia, it is expected that ovigerous females may perform some behaviours to protect their embryos from low oxygen tension. The studies on behaviour of ovigerous crabs upon exposure to hypoxia showed that both non-ovigerous and ovigerous crabs exhibited similar behaviour by agitating and frequently stood with legs extended and ventral surface of the abdomen elevated off the floor of the chamber, then the elevated activity ceased within a few minutes, and the animals became quiescent for the duration of the hypoxic exposure. The quiescent behaviour seemed to be an advantage for adult crabs, since their metabolic rates was reduced and thus they did not die. The developing eggs, however, would gain no direct advantage from this behaviour.

The observations on heart rates and scaphognathite activities found no difference in heart rates and scaphognathite activities between non-ovigerous and ovigerous crabs. This results also correlated with the results on rate of oxygen consumption in ovigerous crabs that the increased rate of oxygen consumption was due to developing eggs only. Thus, in *H. rotundifrons*, both developing eggs and ovigerous females have no special ventilatory mechanism to regulate their use of O_2 during hypoxia. Ovigerous crabs normally buried themselves in moist sand during low tide. Such sand might be quite hypoxic due to the respiration of microorganisms. O_2 would not diffuse into interstitial water more than a few mm unless it was flowing or agitated. Thus, it seems that these crabs and their eggs are likely to encounter hypoxic water during the low tide period. However, in the field, the maximum time for each low tide period is about six hours and actually these crabs experience low tide for not more than three hours since they

inhabitant the lower shore. It is thus possible that developing eggs had the ability to tolerate a few hours of hypoxic conditions during the low tide period.

The ability to tolerate osmotic stress is another important factor for crab eggs to maintain their lives in the intertidal zone. As the eggs are carried under the abdomen, they are presumably in contact with surface water at low tide. This water may be diluted by precipitation and run-off or concentrated by evaporation. The problems are presumably more severe and prolonged for *C. lavauxi*, whose habitat is near the top of the shore. Physiological studies on osmoregulatory of developing eggs of *H. rotundifrons* and *C. lavauxi* revealed that crab eggs of both species maintain hyperosmotic condition in dilute seawater for long periods. Measurements of the four cations by AAS in eggs of both species also demonstrated that internal ions are retained and regulated differently by different stage of eggs. The overall concentrations of all four cations increased with their development and decreased as the salinities decreased. The increase of ions during development suggested that eggs took up ions from the water and they did not contain all the ions needed for development when they were laid. Sodium and potassium were found to be the main cations in the eggs. Sodium was consistently hypo-ionic and potassium hyper-ionic compared with the experimental seawater. Sodium and magnesium concentrations changed at a proportionally greater rate with dilution than did potassium and calcium.

During acclimation of crab eggs to diluted seawater, there was a decrease in the osmotic pressure of eggs. This decrease may be accounted for by changes in the osmotically active solutes (osmolytes) since the total osmolyte in eggs decreased with osmolality. The decrease of ions in eggs with salinities were also found to be corresponded with the decrease of total osmolytes. However, the main osmolytes in the eggs at all stages are non-ionic, presumably free amino acids, yolk products and other organic metabolites. Thus it is possible that these compounds play a role in egg ionic regulation which is similar to isosmotic intracellular regulation found in crab muscle (Gerard and Gilles, 1972).

Apparently, crab eggs have abilities of osmotic and ionic regulation in dilute seawater. However, since eggs, especially at the early stage, possess no regulatory organ, the mechanism used to regulate their internal water and ions was investigated. Water and sodium influx studies revealed that egg membranes of *H. rotundifrons* were very permeable to water and salt throughout their development. Eggs at the early stage are more permeable to water but less permeable to sodium than eggs at the late stage. This is correlated with the survival study that eggs at the early stage had reduced abilities to

survive in dilute seawater. There are at least two components of water and sodium exchange with the seawater in eggs at all stages. This corresponds to the results on membrane study that eggs at both early and late-stages of *H. rotundifrons* are enclosed with membranes which consist of two layers. Eggs of many decapods were also reported to be consisted of two membrane layers (Herrick, 1986; Williamson, 1904; Yonge, 1937; El-Sherief, 1993).

The results on water and sodium influxes thus indicated that eggs do not develop a closed system with an impermeable barrier to the passage of water and ions. Pandian (1970) reported that eggs of *Homarus gammarus* are permeable to water at some stages and water impermeable at others. However, Pandian's method is based on total water content rather than fluxes and cannot provide unequivocal information on permeability. Direct measurements of the internal pressure of *H. rotundifrons* eggs indicated that eggs do not develop a high internal hydrostatic pressure which would oppose the osmotic uptake of water (Taylor & Leelapiyanart, unpublished). Therefore, it appears for eggs to maintain their water and ions in dilute seawater, they must perform osmotic work involving active uptake of ions and active excretion of water. However, the routes for return of water and sodium to the medium require further investigation.

Another important factor that has an influence on survival of crab eggs is the time of hatching. This is probably the most important factor to determine the population of crabs in the ecosystem. Hatching rhythm has been studied in ovigerous crabs entrained to artificial tidal and light/dark (LD) cycles in laboratory. Release occurred in relation to the new LD and tidal cycles in both *H. rotundifrons* and *C. lavauxi.* For *H. rotundifrons*, hatching occurred over 4-6 consecutive nights and there seems to be a complex interaction of hatching with light cycle and tidal cycle. For *C. lavauxi*, larval release is a single event and primarily related to the tides. Release occurring during the period of immersion, regardless of whether it was day or night. In both species, detached eggs were able to hatch into larvae independently of females although percentages of hatching were less. Hatching in both species occurred at the same time as attached eggs. This suggested that embryos within the eggs might determine the time of hatching and females synchronized hatching. However, it is still unknown what mechanisms control hatching in these crabs. Davies (1968) concluded that in decapod crustaceans the egg size increase during incubation was due to either a slow but steady osmotic swelling of the embryo itself. He further suggested the size increase is brought about by uptake of water which increases the internal pressure of the egg up to the time of hatching. Since eggs of *H. rotundifrons* are very permeable to water and salt throughout their development, this mechanism can not provide a full explanation.

In conclusion, survival of developing eggs of both *H. rotundifrons* and *C. lavauxi* during their long incubation period under the abdomen of their mother is due to a combination of physiological and ecological adaptations. On the physiological side, the eggs are resistant to a wide range of external salinities and low oxygen tension. Developing eggs of *H. rotundifrons* are hyperosmotic in dilute seawater and have abilities to survive for a few hours in hypoxic water. This enables eggs to survive in hypoxic conditions or when there is a change in salinity during the low tide periods. The principle ecological adaptation is the timing of larval release. In both species, hatching occurred during summer time, this is perhaps related to the availability of suitable food for crab larvae. Zoea larvae of crabs may feed on phytoplankton which normally bloom during the summer period. The rate of egg development (incubation period) is ecologically important to the timing of larval release into the pelagic environment. Hatching in *H. rotundifrons* occurred in relation to both LD and tidal cycles, whereas it is related to tidal cycle in *C. lavauxi.* The night-time larval release by *H. rotundifrons* in small batches over a period of time may be to avoid visual predators and to spread the risk of predation. The relation of the time of hatching to tide cycles may be an adaptation to maximize the possibilities that the zoea larvae will be transported by tidal currents into the sea.

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